Imperial College London

PATHOGEN DIVERSITY AND HOST RESISTANCE IN DIEBACK DISEASE OF COCOA CAUSED BY *FUSARIUM DECEMCELLULARE* AND *LASIODIPLODIA THEOBROMAE*

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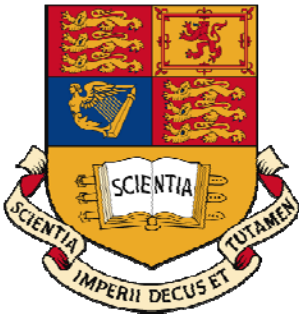
March 2009



Thesis presented for the degree of Doctor of Philosophy at Imperial College of Science, Technology and Medicine, and for the Diploma of Imperial College (DIC)

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Abstract

Dieback disease caused by *Fusarium* and *Lasiodiplodia* species is a major threat to cocoa production in Ghana and elsewhere in West Africa. Current recommendations involve insecticide application to control mirid bugs whose feeding punctures provide entry points for these fungi. Little is known about the true identity of the causal pathogens of this disease. Earlier work implicated *F. decemcellulare* as the causal agent and more rarely *L. theobromae* (Cotterell, 1927; Crowdy, 1947). A total of 117 single spore fungal cultures was established from diseased cocoa stems imported from Ghana. On morphological grounds cultures could be designated as either *Fusarium* or *Lasiodiplodia* spp. The *Fusarium* cultures exhibited inter-isolate variability with respect to macroscopic appearance and macro-conidium morphology, suggesting the presence of more than a single species. The isolates were further characterised by PCR amplification and sequencing of the ITS region of rDNA and comparison with authentic reference cultures. Thirty-seven *Fusarium* isolates were identified to twenty *F. chlamydosporum,* nine *F. solani* and four isolates each of *F. oxysporum* and *F. proliferatum.* The thirty-six *Lasiodiplodia* isolates were identified to two species, twenty-seven *L. pseudotheobromae* and nine *L. theobromae*. In pathogenicity tests, *F. chlamydosporum*, *F. oxysporum*, *F. proliferatum*, *F. solani* and *L. pseudotheobromae*, previously unknown as pathogens of either cocoa or any member of the Malvaceae, caused significant wilting and dieback in Amelonado seedlings similar to that observed in the field. All isolates exhibited optimal growth at 30 ºC on PDA. Disease incidence in 29 and 15 cocoa germplasm lines in the laboratory and greenhouse, respectively, showed reproducible differences in their reaction to necrotic lesion and dieback infection. LCTEEN 37/F was one of the most susceptible genotypes. CATIE 1000, T85/799 and MXC 67 were the most tolerant and could be used in cocoa breeding programmes for resistance to dieback.

DECLARATION OF OWN WORK

I confirm that this thesis:

Pathogen diversity and host resistance in dieback disease of cocoa caused by *Fusarium decemcellulare* and *Lasiodiplodia theobromae*

is entirely my own work, conducted under the supervision of Dr Simon Archer and Dr Simon Leather. Where any material could be construed as the work of others, it is fully cited and referenced, and or with appropriate acknowledgement. No part of this research has been submitted in the past, or is being submitted, for a degree or examination elsewhere. The input of my supervisors to the research and to the thesis was consistent with normal supervisory practice. I grant copyright of this thesis to Imperial College London.

March 2009

Name of student: Richard Kwame Adu-Acheampong

Name of supervisor: Dr Simon Archer

Acknowledgements

It is a pleasure to take a few moments and remember all of the support and assistance that I received as I worked through this thesis. Friends, family, supervisors, colleagues, and many others have sustained me throughout the process. I was fortunate to have been offered a scholarship by the Commonwealth Scholarships Commission of the United Kingdom, to whom many thanks are due. I am grateful to the late Dr M. R. Appiah, former Executive Director of the Cocoa Research of Ghana, for granting me permission to carry out the study. Two excellent advisors, the late Dr Beatrice Padi and Dr F. K. Oppong, encouraged and challenged me every step of the way. I wish also to thank Drs Y. Adu-Ampomah, F. K. Amoah, J. B. Ackonor, I. Y. Opoku, A. Y. Akrofi, Boamah-Adomako, Michael Assuah and the entire staff of the Entomology Division at CRIG for their stimulating support. Dr Andrew Daymond of University of Reading is acknowledged for the provision of budwood of cocoa clonal materials, likewise Dr Gary J. Sammuels (USDA) for the supply of *Fusarium decemcellulare* reference isolates and Rob Lockwood for useful references. I am indebted to my supervisors Dr Simon Archer and Dr Simon Leather for their support, continued interest and advice, and stimulating suggestions and encouragement that helped me during my entire stay at Silwood Park. For all of their support, I thank the following technicians, Paul Nicholas, Jim Culverhouse, Frank Wright, Paul Beasley and Glen Chalker. I am particularly grateful to the following persons from the Biology Division, Sandy Whitehead, Sue Lomas, Nabeil Salama, Biswanath Das and Hanno Schäfer for their help even by email and telephone and often at short notice. I am specially obliged to Dr Robert Coles Brian (formerly at Jealott's Hill Research Station [I.C.I. Ltd, UK] and Ms Janet McLennan (of Sunningdale Baptist Church, and formerly Deputy Head of Coworth Park School, UK) who critically read the text. Coincidentally, Prof. Stephen Henry Crowdy who first investigated this disease problem in Ghana (then Gold Coast) worked alongside Dr Brian at Jealott's Hill for 30 years. Janet looked closely at the final version of the thesis for English style and grammar, made corrections, and offered suggestions for improvement. Finally I would like to give my special thanks to my wife Beatrice whose patient love enabled me to complete this work. This work was conducted under the Department for Environment, Food and Rural Affairs (DEFRA) Plant Health Licence No. PHL 189B/5316(03/2006).

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Abbreviations

acre	ac
active ingredient	a.i.
aqueous concentrate	a.c.
boiling point	b.p.
base pair	bp
Celsius	°C
Commonwealth Agricultural Bureau International	CABI
correlation coefficient	r
diameter	diam.
gram	g
hour	h
hectare	ha
internal transcribed spacer	ITS
kilogramme	kg
least significant	LSD
less than	<
litre	l
microlitre	µl
micrometre	µm
milligramme	mg
millilitre	ml
millimetre	mm
minimum	min.
minute	min
MMT	million metric tons
more than	>
polymerase chain reaction	PCR
percentage	%
probability value	p
relative humidity	r.h.
revolutions per minute	rpm
second	s
species (singular)	sp.
species (plural)	spp.
square metre	m^2
square millimetre	mm^2
standard deviation	s.d.
standard error of the difference of means	s.e.
Sustainable Perennial Crops Laboratory	SPCL
ultra-violet	u.v.
United States Department of Agriculture	USDA
variety (botanical use)	var.
volume	vol.

1 Chapter 1

1.1. Literature review

1.1.1. Origin, history, geographic distribution and diseases of cocoa

Cocoa originated in the foothills of the Andes in the Amazon and Orinoco basins of South America and has been consumed in Mesoamerica since at least 600 BC (Hurst *et al*., 2002). Cocoa was introduced into Central America by the ancient Mayas, and cultivated in Mexico by the Toltecs and later by the Aztecs. It is now grown predominantly in West Africa, Latin America and Southeast Asia (Cheesman, 1944; Motamayor *et al*., 2003; Sereno *et al*., 2006).

Archaeologically, the Maya traders, who developed cocoa as a domesticated crop, drank cocoa as early as 400 BC in Costa Rica, and they believed the tree was a gift from a god. A drink from the roasted beans, often used in ceremonies and rituals, was called xocolati, from which the word 'chocolate' was derived. There is also extensive documentation of medicinal uses of cocoa that can be traced to ancient Aztec documents. A surviving document, the Florentine Codex compiled in 1590 by a Spanish priest extensively documents cocoa-based preparations and the illnesses that they were used to prevent or cure (Bennett, 2003).

Cocoa is believed to have been introduced into Trinidad in 1525, into Southeast Asia in 1560 and westwards from Acapulco to the Philippines in 1614 (Wood and Lass, 2001). According to this author, by 1700, cocoa was being grown throughout Central America and on many of the islands in the Caribbean, as well as in areas adjacent to the Andes in South America.

Amelonado type cocoa was sent from the Amazon Basin of Brazil to Bahia in 1746 and to West Africa in 1822 at Principe, from where it was taken to Sao Tomé and Fernando Po (now Bioko) in 1830 and 1854, respectively (Wood and Lass, 2001). Nigeria and Ghana received their seed from Fernando Po in 1874 and 1879,

respectively, while Sri Lanka (then Ceylon) had her first and second introductions in 1834-35 and 1880 from Trinidad. The cocoa from Ceylon was said to have been distributed to Singapore and Fiji in 1880, to Samoa in 1893, to Queensland, Australia, in 1886 and to Bombay in 1887. The Ceylon material was also introduced into Zanzibar and then German East Africa (Tanganyika) in 1887 and 1893, respectively, and later, into Madagascar (Wood, 1991).

1.1.2. Biology and ecology of cocoa

Cocoa is an understorey forest plant that grows in the wet, humid tropics within latitudes 20° north and south of the equator, usually below 300 m (Cobley and Steele, 1976). The plant is particularly adapted to uniformly high temperatures, minimum 13 °C and maximum 33.5 °C (mean = 26 °C), mean annual rainfall of 1000 to 3000 mm and deep well-drained soils, free from iron concretions but high in nutrient content and organic matter (Hartemink, 2005). The prevailing weather condition at Tafo within the cocoa-growing belt of the Eastern Region of Ghana is shown in Figure 1.

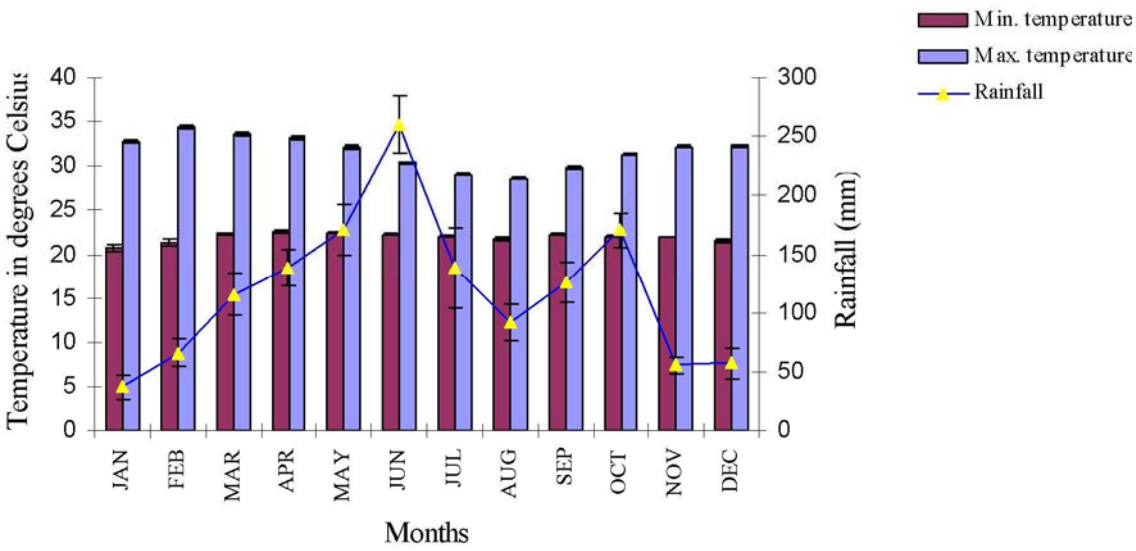

Figure 1. Cocoa Research Institute of Ghana, Akim-Tafo weather: 11-year averages and standard errors 1997-2007.

Reed (1976) established that cocoa is a cauliflorous (i.e. it produces flowers on the older branches or main stem) (Figure 2) and semi-deciduous plant usually reaching 5

to 10 m high. At 1 to 1.5 m, the terminal bud breaks into 3 to 5 meristems to give rise to several lateral branches (Figure 3). Vegetative shoots arising from the base of the main trunk (chupons) have leaves arranged in ⅝ pattern of leaf arrangement (phyllotaxy) while lateral branches have ½ pattern of arrangement (Figure 4). The petiole has two joined cushion-like swellings (pulvini), one at the base and the other at the point of insertion of the leaf. Stipules are two and deciduous. The leaf blade (lamina) is elliptical-oblong or obovate, simple, 10 to 60 cm long, 4 to 20 cm broad; generally smooth, sometimes hairy, rounded and obtuse at the base with a pointed apex. The roots are mostly a mass of surface-feeding roots, with the taproot penetrating to 2 m in friable soil, less deeply when compacted. The arrangement of flowers on the stem is dichasial (i.e. two lateral flowers originating from opposite points beneath the flower stalk). The primary peduncle is very short, often thick and lignified. The flower peduncle is 1 to 4 cm long and there are five sepals, which are triangular and whitish or reddish in colour.

The five petals are joined at the base into a cuplike structure. They are whitish-yellow with dark purple bands at the underside; ligules are spathulate and yellowish. There are five fertile stamens alternating with staminodes, the two whorls uniting to form a tube. There are two anthers each about 0.4 mm long, with fused stamens. The ovary is superior with a single style terminating in five sticky stigmatic surfaces. The pods are indehiscent and variable in size and in shape. They range from 10 to 32 cm long, and may be ovoid, near spherical, oblong, pointed or blunt at the base. The outer surfaces of pods usually have 5 to 10 furrows. Unripe pods are green, red or purple, ripening to yellow, red or purple and contain 20 to 60 seeds (beans) per pod, arranged in five rows, variable in size, 2 to 4 cm long, and 1.2 to 2 cm broad, ovoid or elliptic. Placentation is axile with seeds embedded in mucilage, flat or round with white or purple cotyledons; seeds per kg range from 625 to 1125. It has a complex system of self-incompatibility. After successful pollination, fertilization takes place within 36 hours; the sepals, petals and staminodes drop away and the stamens and pistil wither. The young pod, known as the cherelle, begins to develop by longitudinal elongation, followed by increase in width. It takes five months or so for a pod to develop from fertilization to maturity depending on the variety (Glendinning, 1972). Flowering in Ghana occurs at definite peak periods. It is low and irregular in the January to March dry season, then very heavy at the beginning of the rains usually in April. It then

declines to become almost nil during the peak of the main crop. Thus, in Ghana, the crop ripens in two main periods, 'minor crop' roughly from mid-May to mid-July, and the 'main crop' from September normally to December and sometimes to February. Currently, where weather conditions are more uniform throughout the year, cropping of some newer varieties may be almost continuous.

Figure 2. Cocoa flowers developing from cushions on a main stem. Photo: R. A-A.

Figure 3. The main terminal bud forms three to five branches (the jorquette) that grow out as fan branches. Photo: R. A-A.

Figure 4. Leaf arrangement on cocoa stem: ⅝ pattern (A); ½ pattern (B). Photo: R. A-A

1.1.3. Pollinators

The cocoa plant is naturally out-breeding (Wood and Lass, 2001). Although there is general belief that small insects are the pollinating agents, there is no general agreement as to which insects are primarily responsible. Many authorities credit midges, especially *Forcipomyia quasiingrami* Macfie and *Lasiohela nana* Macfie (Macfie, 1944; Chatt 1953; Saunders, 1959; Barroga, 1964; Fontanilla-Barroga, 1965; Kaufman, 1973) while others believe it is mainly by ants (e.g. *Crematogaster* spp.), aphids (*Aphis gossypii* Glover and *Toxoptera* spp.), thrips (*Frankliniella parvula* Hood), and wild bees (Jones,1912; Harland, 1925; Cope, 1940; Voelcker, 1940; Billes, 1941; Posnette, 1942; Muntzing, 1947; Posnette and Entwistle, 1957; Urquhart, 1961;).

1.1.4. Taxonomy of cocoa

The genus *Theobroma* has about twenty-two species, but only *Theobroma cacao* L. is grown on a large scale (PMN No. 12, 2001). *Theobroma cacao* until recently belonged to the family Sterculiaceae (Wood and Lass, 2001) and was divided into three distinct groups: Forastero, Criollo and Trinitario based on the morphology of the pod (Figure 5). The first comprises 95 % of the world production of the crop and is the most widely used (Amoah, 1995). Forastero cocoa is vigorous and the most resistant to diseases (Lerceteau *et al.*, 1997) but the highest quality cocoa comes from the Criollo, which is unfortunately, poor yielding, highly susceptible to pests and diseases and difficult to produce. Consequently, very few countries produce it, with the greatest production coming from Venezuela. Trinitario cocoa is considered to have originated by natural hybridization between the first two groups and has different characteristics including good aromatic pods and beans (seeds) (Lerceteau *et al.*, 1997; Motamayor *et al.*, 2003; Sereno *et al.*, 2006). The Criollos have elongated, ridged, and pointed pods whereas the Forasteros have short, roundish, and smooth pods. Forastero cocoa is further divided into Lower and Upper Amazon Forastero according to its geographical origin. The classification of some cocoa clones, however, remains uncertain: for example, the cocoa identified as Nacional from Ecuador and classified as Forastero (Cheesman, 1944; Soria, 1970) was recently placed among the Criollos (Enriquez, 1992). A number of subspecies and forms of

cocoa have now been recognized, from which a great number of cultivars have been developed. Several cultivars are named according to the place where they were developed.

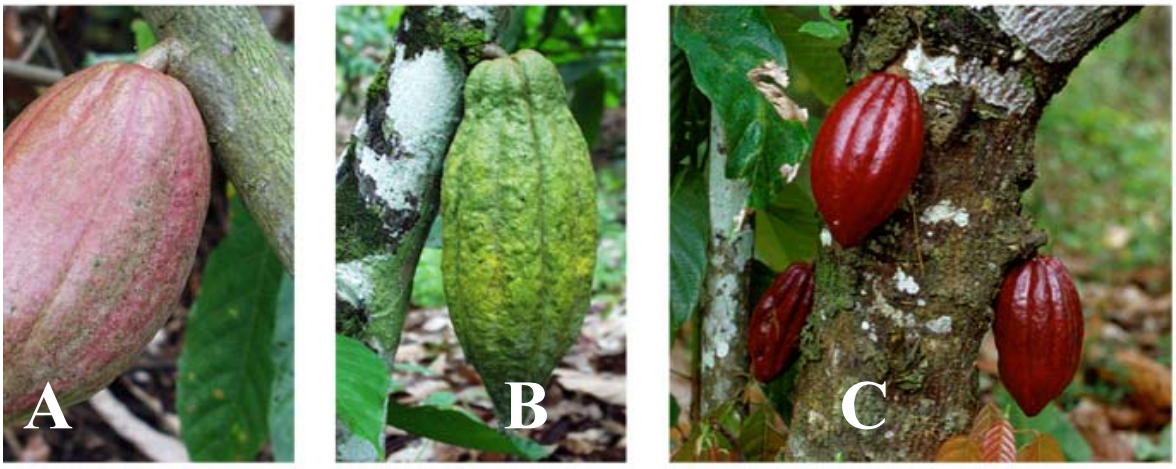

Figure 5. Morphology of pods from different cocoa types: A, Forastero; B, Trinitario; C, Criollo. Source: Cocoa Atlas 2002.

Efforts are being made to reduce the number of botanical descriptors used in cocoa identification in order to facilitate genotype characterization (Lerceteau *et al.*, 1997). The use of molecular data for systematics in cases where morphological characters are conflicting or missing has been addressed by several authors. Based on isozyme data, Upper Amazonia is considered the primary centre of diversity (Lanaud, 1987; Warren, 1994). Laurent *et al.* (1994) observed mitochondrial, chloroplastic, ribosomal and cDNA polymorphisms and were able to provide some insight into the evolution of cocoa, underlying the complementary aspects of all these markers. Random amplified polymorphic DNA (RAPD) technology (William *et al.*, 1990) has also been successfully used to infer genetic relationships within many species, including *T. cacao* (Figuera *et al*., 1994; N'goran *et al.*, 1994). After a study of the genetic variability among cocoa accessions using RAPD and RFLP markers, Lerceteau *et al.* (1997) concluded that the Nacional type cocoa was genetically distinct and different from well-known types such as Forastero, Criollo and Trinitario.

Recent phylogenetic investigations on the plastid *atp*B, *ndh*F and *rbc*L sequences, morphological, and chemical characterisation have suggested the inclusion of the

Sterculiaceae into the distinct family Malvaceae (Alverson *et al*., 1999; Bayer *et al*., 1999; Judd and Manchester 1997; Whitlock *et al*., 2001). The most parsimonious trees derived from the nucleotide sequences corroborated the sectional classification of *Theobroma* and *Herrania*, which had been based on morphology (Figure 6).

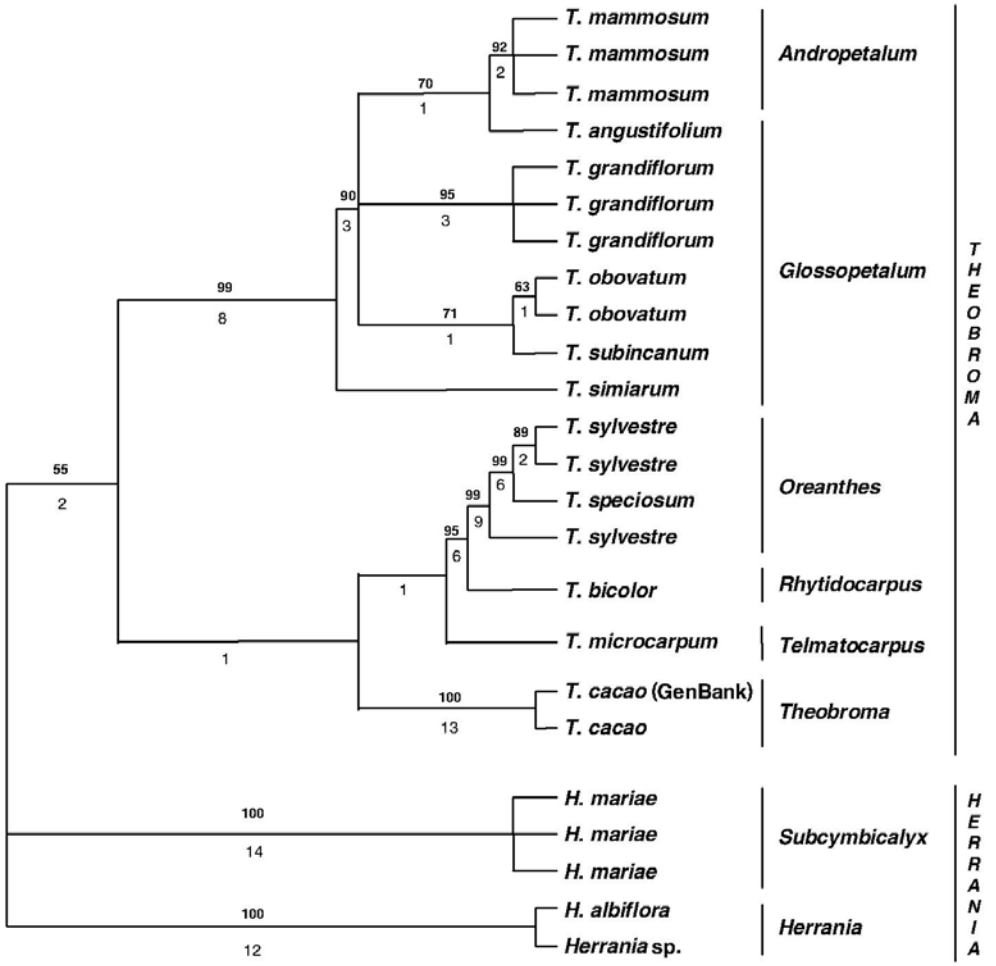

Figure 6. Strict consensus of 16 most parsimonious trees generated from nucleotide sequence of the trypsin inhibitor gene from *Theobroma* and *Herrania* species. Length¼273 steps; C.I.¼0.7839; H.I.¼0.2161; R.I.¼0.8628; R.C.¼0.6763. Percent of 1,000 bootstrap replications is given above the branches. Decay index values are shown below the branches. Source: Silva and Figueira (2005).

1.1.5. Cultivation

Cocoa cultivation has been examined in some detail by a number of authors (Amoah, 1995; Wood and Lass, 2001). The crop is traditionally grown in cleared forest following logging, and is inter-cropped with food crops during the first three years (Figure 7). Cocoa has historically been grown from seed with the seed generally grown in a nursery in polybags to produce seedlings or they are sown directly. The seeds germinate within 7 to 10 days and do not pass through a dormancy period. Recently, alternative propagation approaches have been proposed. Vegetative propagation is becoming popular as the genetics of the tree are then known, and can be controlled. This is also desirable because bean quality and size, time to fruit bearing and productivity of trees are improved. Budded trees for example, are smaller than trees produced from seed. Consequently, more trees per hectare can be planted, and the pods are borne closer to the ground to aid harvest.

Figure 7. A new cocoa farm showing food-crops planted to protect the young plants from direct solar radiation. Photo: USDA, SPCL.

When the seedlings are six months old, or have reached about 0.6 m tall, they are transplanted into shaded fields at 2.5 m × 2.5 m or 3.0 m × 3.0 m apart (Amoah, 1995). Plantain appears to meet most of cocoa's shade requirements in this respect, whereas bananas compete heavily for moisture during the dry season. Intercropping of cocoa with food crops such as maize, cassava, cocoyam, yams, plantain and coconut is a normal practice in W. Africa. Cocoa trees respond to fertilizers especially in the absence of shade. The common fertilizers and their doses are 100 g of N, 40 g of P_2O_5 and 140 g of K_2O per plant per year in 2 split doses. Trees younger than three years require only half or three-fourths of these rates.

Farm husbandry such as weeding and temporary shade is essential during the first three to four years. Average yields range from 0.5 to 10 kg per tree (Duke, 1983) and although pods mature throughout the season, there are two main crops in a year in West Africa: in September-January and in April-June. The original art of hand harvesting the pods, using the traditional long handled sickles, is still practised today in Ghana when they are ripe or have achieved a yellow colour (for most varieties) (Figure 8) The harvested pods are collected, broken open, usually with a wooden baton and the wet beans (covered with sweet mucilage) removed by hand. The fresh beans are fermented for up to 6 or 7 days on plantain or banana leaves, and then covered with more leaves to aid the development of the chocolate flavour when they are roasted in the factory. Fermentation usually is done under shade (away from direct sunlight) and larger heaps (i.e. more than 90 kg) are turned after three days to ensure uniform conditions throughout the heap. Perforated plastic sheets and wooden boxes are sometimes used in the absence of plantain or banana leaves. After fermentation, the beans are sun-dried on raised bamboo, palm or raffia mats (Figure 9) for 7 to 14 days or longer on cloudy/rainy days to the desired 7.5 % moisture content.

Figure 8. Mature mixed hybrid cocoa tree bearing ripe yellow pods. Photo R. A-A.

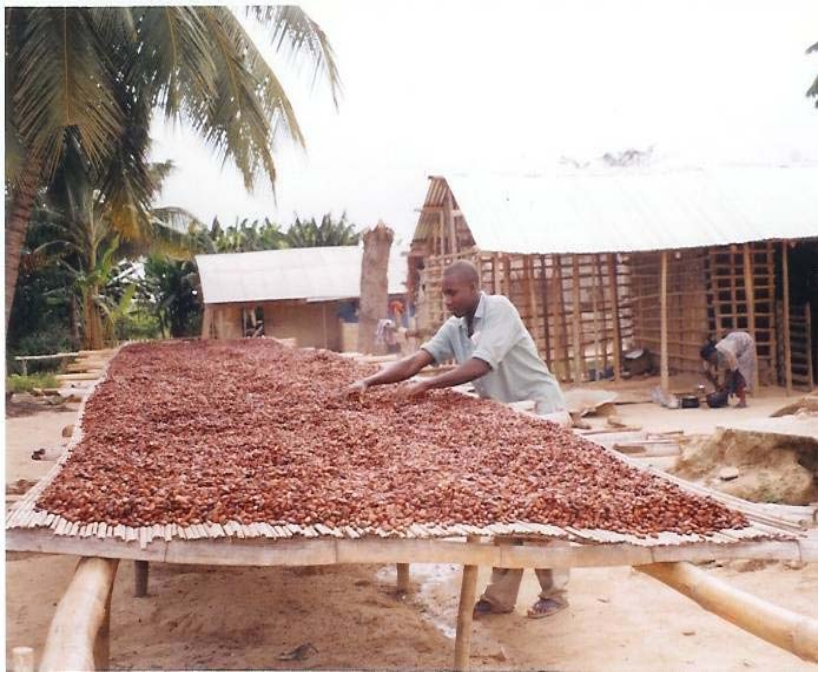

Figure 9. Sun drying of fermented cocoa beans by a Ghanaian cocoa farmer. Photo: R. A-A

1.1.6. World cocoa production

The world cocoa trade is worth about $30 billion annually and this has grown at a rate of about 5 % per year in the past few years (ICCO, 2005). Almost 4 million metric tons (MMT) of beans were produced in 2005, and the 2.7 MMT that were traded internationally in 2003 were worth $4.2 billion. In 2001, the global chocolate market was worth $73 billion (Ploetz, 2007). In 2003 and 2004, global production of dry cocoa beans reached an all time record of 3.5 million tons. At present, African countries account for about 80 % of the world cocoa export (Figure 10 and Figure 11) with the Ivory Coast alone accounting for 34 % (Table 1). In Ghana, cocoa and cocoa products account for two-thirds of export revenue and in the forest belt (Figure 12), its importance as a cash crop is paramount.

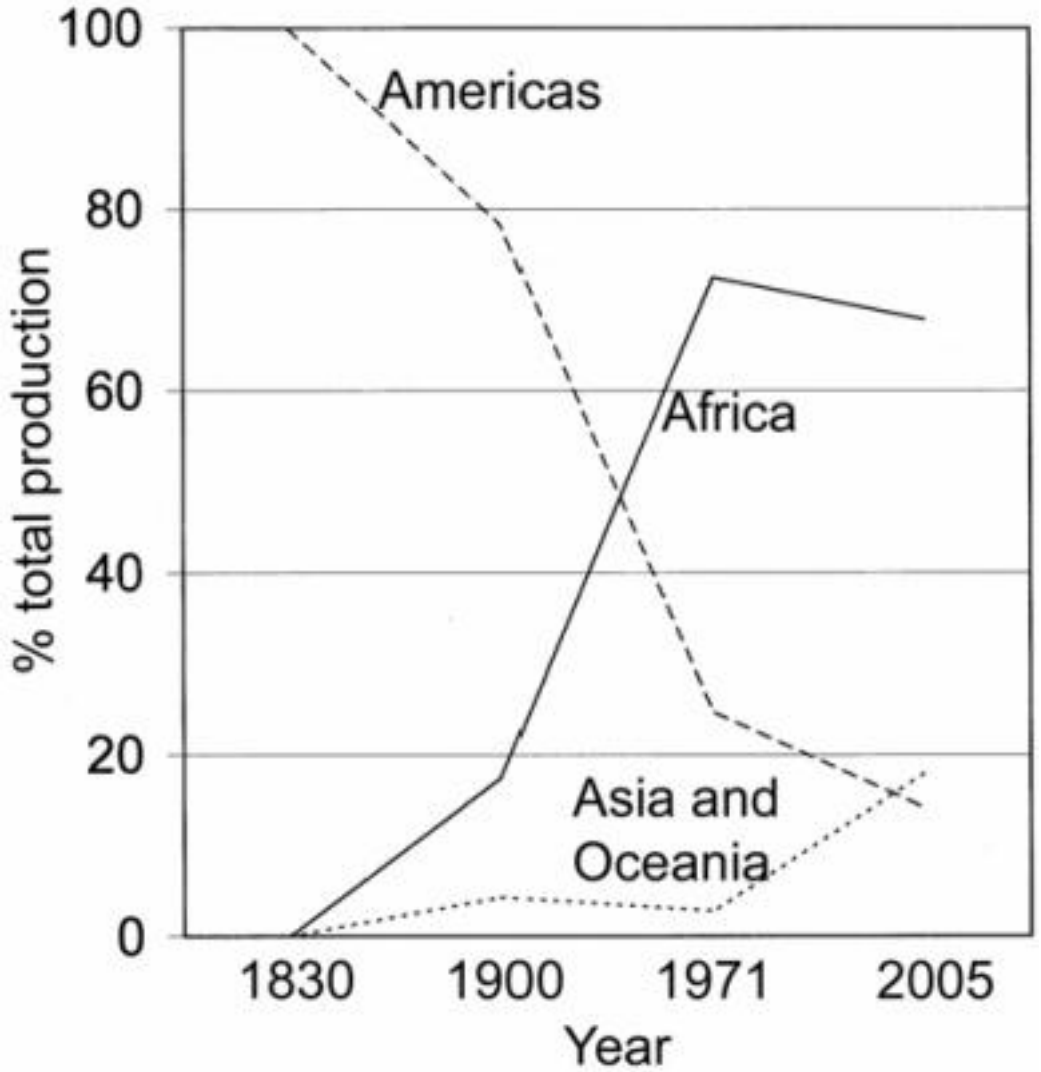

Figure 10. Regional trends in global cocoa production since 1830.
Source: (Ploetz, 2007).

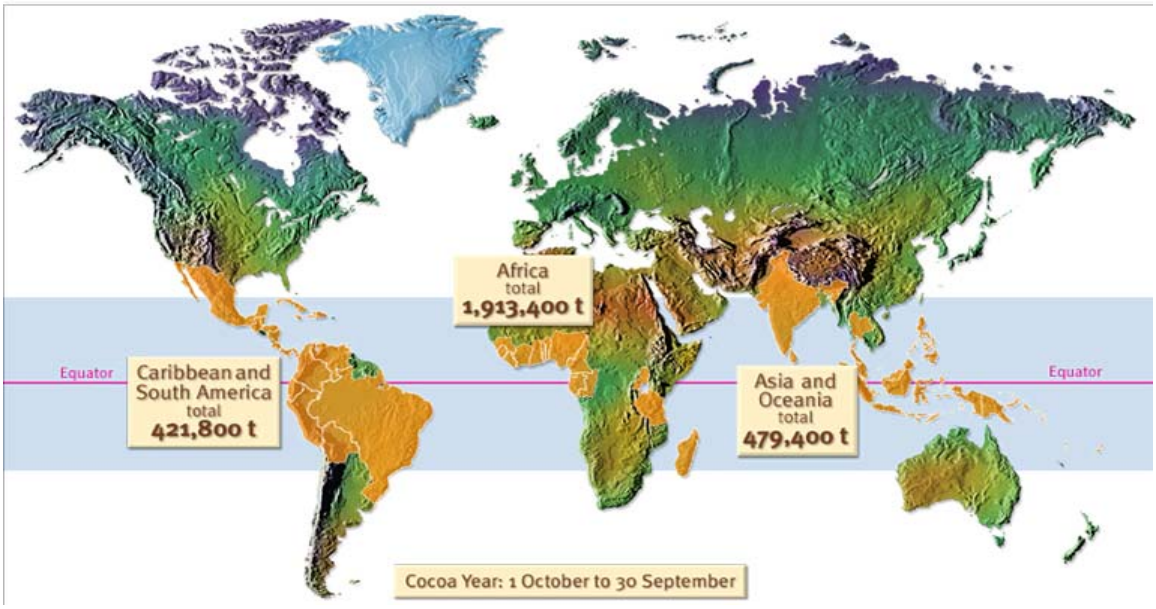

Figure 11. World cocoa production figure for year 2002, Source: World Cocoa Foundation.

Figure 12. Map of Ghana showing the cocoa growing areas.

Table 1. The ten most important cocoa producing countries in year 2005. Source: Ploetz, 2007.

Country	Production (MT)	% Total
Ivory Coast	1,330,000	33.9
Ghana	736,000	18.8
Indonesia	610,000	15.5
Nigeria	366,000	9.3
Brazil	214,0774	5.5
Cameroon	180,000	4.6
Ecuador	137,178	3.5
Colombia	55,298	1.4
Mexico	48,405	1.2
Papua New Guinea	42,500	1.1
World Total	3,923,183	Other: 5.2
		Africa: 67.85
		Asia and Oceania: 18.08
		Western Hemisphere: 14.06

In Ghana, smallholder farmers often with 1 to 2 hectares (ha), grow cocoa under extensive management systems (Amoah, 1995; Padi and Owusu, 2001). Some 800,000 smallholder farmers in Ghana grow cocoa on an area of about 1,268,000 ha of land and it is estimated that over 10 million people in West Africa are supported by some 1.2 million independent family cocoa farms. Consequently, there was an initiative by the Ghana Government and the Ghana COCOBOD in 2001 to help farmers with the supply of planting material, labour-efficient cropping systems, pest and disease control, and harvesting, fermentation and drying.

1.1.7. Major diseases of cocoa

Since 1879, cocoa has occupied a place of economic importance in Ghana and it is the crop with the largest total acreage in the forest belt of the country (Amoah, 1995) (Figure 12). Cocoa is also a key foreign exchange earner to many West African

economies (Amoah, 1995). However, many diseases and pests affect cocoa, with some estimates putting losses as high as 30 to 40 % of global production. There are many hundreds of insect pests and pathogens recorded (Entwistle, 1972; Purdy *et al.,* 1998; Aime and Phillips-Mora, 2005). Of these, only a fraction is economically important, and diseases rather than insects, are the biggest problem (PMN No. 12, 2001). Thorold (1975) has extensively covered the major diseases of cocoa in an excellent book. He categorises cocoa diseases according to the plant tissues involved such as diseases of stems, trunk, leaves, pods, flowers and roots among others.

1.1.7.1. Dieback disease

In addition to fungal pathogens, non-pathogenic saprophytic fungal associates of cocoa have been found as epiphytes or endophytes, many of which have the potential to become parasites when conditions favour increased aggressiveness (Purdy *et al*., 1998). Arnold and Herre (2003) reported that more than 800 strains and more than 500 morphospecies of endophytic fungi were isolated from cocoa in Panama. Turner (1967) reported the presence of more than 80 species of fungi associated with the dieback of cocoa trees in West Africa. None of these was said to be a primary pathogen of cocoa, but all had been reported as opportunistic species that attacked cocoa stressed by insects such as mirids.

Mirid and fungi-related dieback accounts for losses of millions of dollars each year in West Africa (Padi and Owusu, 2001). Strategies to combat these losses based on finding or broadening plant resistance to pathogens have received little attention. The disease has been linked to at least seven causal organisms, but most records centre on two fungus species, *Albonectria rigidiuscula* (Berk. & Broome) Saccardo [anamorph: *Fusarium decemcellulare* Brick] and *Botryosphaeria rhodina* (Berks & Curtis) von Arx [anamorph: *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.] (Owen, 1956) (Tables 2 and 3). Most literature refers to the anamorph names which are used routinely in this thesis.

Table 2. Taxonomy of *Albonectria rigidiuscula* (formerly *Calonectria rigidiuscula* anamorph: *Fusarium decemcellulare.*

Albonectria rigidiuscula
Scientific classification

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Sordariomycetes
Subclass:	Hypocreomycetidae
Order:	Hypocreales
Family:	Nectriaceae
Genus:	*Albonectria*
Species:	***A. rigidiuscula***

Binomial name
Albonectria rigidiuscula (Berk. & Broome) Rossman & Samuels, (1999)
Synonyms
Calonectria eburnea Rehm, (1888) *Calonectria lichenigena* Speg., (1889) *Calonectria rigidiuscula* (Berk. & Broome) Sacc., (1878) *Calonectria sulcata* Starbäck, (1899) *Calonectria tetraspora* (Seaver) Sacc. & Trotter, (1913) *Fusarium decemcellulare* Brick, (1908) *Fusarium rigidiusculum* W.C. Snyder & H.N. Hansen, (1945) *Fusarium spicariae-colorantis* Sacc. & Trotter ex De Jonge [as 'spicariae-colorantis'] *Nectria rigidiuscula* Berk. & Broome, (1873) *Scoleconectria tetraspora* Seaver, (1910) *Spicaria colorans* De Jonge, (1909)

Table 3. Taxonomy of *Botryosphaeria rhodina* (anamorph *Lasiodiplodia theobromae*- formerly *Botryodiplodia theobromae*).

Botryosphaeria rhodina

Scientific classification

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Dothideomycetes
Subclass:	Incertae sedis
Order:	Botryosphaeriales
Family:	Botryosphaeriaceae
Genus:	*Botryosphaeria*
Species:	***B. rhodina***

Binomial name

Botryosphaeria rhodina (Berk. & M.A. Curtis) von Arx, *Gen. Fungi Sporul. Cult.* (Lehr): 143 (1970)

Synonyms

Botryodiplodia gossypii Ellis & Barthol., *J. Mycol.* 8: 175 (1902).
Diplodia cacaoicola Henn., *Engler's Bot. Jahrb.* 22: 80 (1895).
Diplodia gossypina Cooke, *Grevillea* 7 (no. 43): 95 (1879)
Diplodia natalensis Pole-Evans, *Transvaal Dept. of Agricult. Sci. Bull.* 4: 15 (1911) [1910].
Physalospora fusca N.E. Stevens, *Mycologia* 18: 210 (1926).
Physalospora glandicola N.E. Stevens, (1933).
Physalospora gossypina F. Stevens
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Botryodiplodia theobromae

Lasiodiplodia theobromae

The association of *F. decemcellulare* and *Lasiodiplodia* with dieback of cocoa was first described by Crowdy (1947). The two important mirid species (Heteroptera: Miridae) in West Africa are *Sahlbergella singularis* Hagl. and *Distantiella theobroma* (Dist.) which feed on shoots and pods. If the feeding punctures become infected with *F. decemcellulare* or *L. theobromae*, large shoots and trees may be killed. As noted by Crowdy (1947), neither the insect nor the fungus alone normally does serious damage to the tree, but in combination, the damage is one of the serious problems facing the

cocoa industry in West Africa. The most conspicuous symptoms of *F. decemcellulare* and *Lasiodiplodia* damage occur on shoots beginning with chlorotic mottling of leaves and necrosis and defoliation proceeding to vascular browning of stems (Entwistle, 1972). In some cases, leaf symptoms are less frequent but stems may still be infected.

There is currently no recommended control for dieback of cocoa. The spores of *F. decemcellulare* are known to survive in wood for years and can be moved accidentally in contaminated tissue (Crowdy, 1947). Synthetic insecticides against mirids came into commercial use in the 1940s (Owusu-Manu, 1990; 1995). Since then several hundreds of other compounds with diverse modes of action covering all the major insecticide groups have become available for use on cocoa. However, global pest management strategies are currently focused on environmental protection and human well-being, and chemical spraying of crops is discouraged wherever possible. Moreover, the world market demand for high quality and residue-free cocoa, together with increasingly strict governmental restrictions on pesticide use, has increased the pressure to develop more effective and sustainable disease control methods.

Conventional methods to detect or isolate the causal disease pathogens, *F. decemcellulare* and *L. theobromae*, have been described by Crowdy (1947) and Owen (1956) and visual disease assessment is based on the recognition of the usual symptoms of dieback such as wilting of leaves and branches. Also at present, no reliable and rapid molecular methods to detect these pathogens in cocoa have been reported.

1.1.7.1.1. Taxonomy and life cycle of Fusarium decemcellulare and Lasiodiplodia theobromae associated with dieback disease of cocoa

Fusarium species are common soil-borne fungi usually found in natural and cultivated soils (Nelson *et al*., 1983). Various species attack perennial crops in the tropics (Ploetz, 2007). For example, the formae speciales of *Fusarium oxysporum* cause wilt diseases in more than 100 plant species (Armstrong and Armstrong, 1981) while *F.*

solani attacks at least 111 plant species belonging to 87 different genera (Kolattukudy and Gamble, 1995). *Fusarium decemcellulare* Brick (teleomorph: *Albonectria rigidiuscula*) causes green-point-gall and dieback of cocoa (Crowdy, 1947; Booth 1971). It is a weak parasite and requires wounds to infect cocoa (Holliday, 1980). To date, most descriptive information on *F. decemcellulare* is based on morphological characters such as the size and shape of the conidia, the presence or absence of chlamydospores, and the structure of conidiophores (Windels, 1992). Potato dextrose agar (PDA) cultures (Figure 13) were used to assess pigmentation and colony morphology of different *Fusarium* species (Summerell *et al*., 2003). The pink to red pigmentation together with yellow sporodochia and the large size of the macroconidia are definitive characters of this species. *Fusarium decemcellulare* also affects avocado (Darvas and Kotze, 1987) and mango (Ploetz *et al.*, 1996).

On cocoa, *F. decemcellulare* attack results from complex interactions with other pathogens and pests. Homothallic and heterothallic isolates of the rare teleomorph, *Albonectria rigidiuscula*, have been reported on cocoa, but only heterothallic strains are known to cause disease (Holliday, 1980). Perithecial isolates on cocoa were homothallic and non-pathogenic (Ford *et al*., 1967). Recently, a dichotomous key on this species and other related Nectria-like fungi was published (Watling *et al*., 2002). The present nomenclature is shown in Table 2.

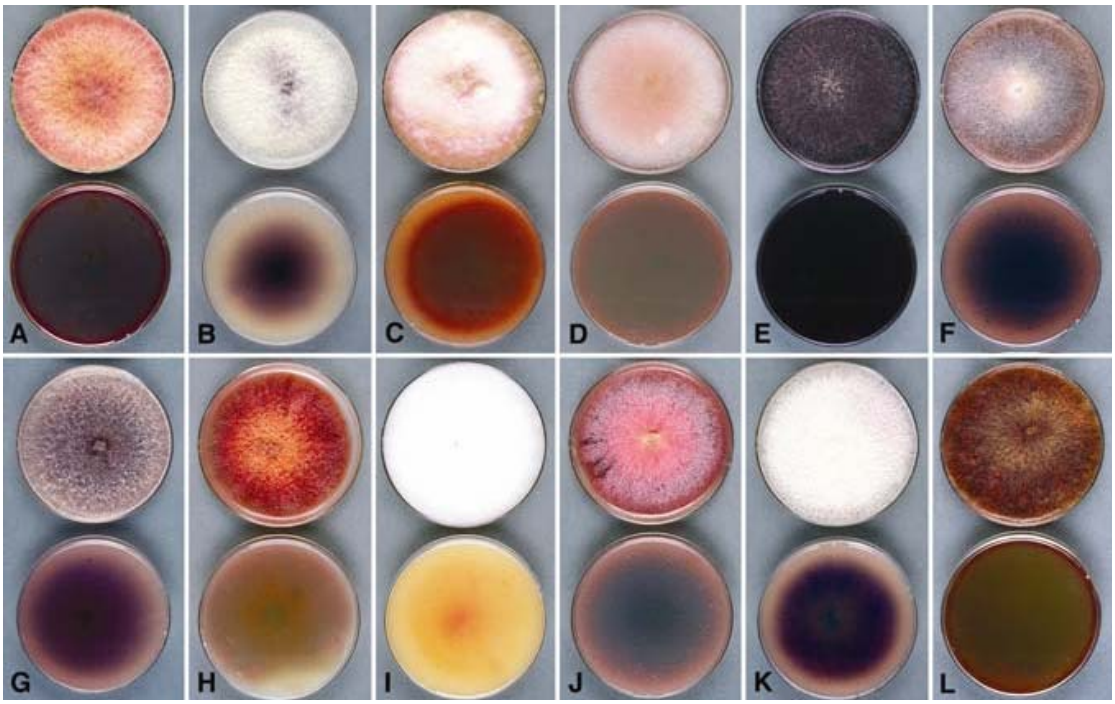

Figure 13. Colony morphology of *Fusarium* species on potato dextrose agar. The top plate in each pair is the upper surface and the lower plate is the under surface. A, *F. poae.* B, *F. oxysporum.* C, *F. acuminatum.* D, *F. nelsonii*. E, *F. subglutinans.* F, *F. nygamai.* G, *F. pseudonygamai*. H, *F. lateritium.* I, *F. thapsinum.* J, *F. decemcellulare*. K, *F. verticillioides.* L, *F. culmorum*. Source: Summerell *et al*., 2003.

Lasiodiplodia theobromae is also an important fungal pathogen of higher plants from tropical and sub-tropical regions (Cardoso and Wilkinson, 2008). The fungus is ubiquitous and able to infect over 500 plant species, causing symptoms ranging from seed rot to the discolouration and dieback of timber (Punithalingam, 1976). The affected plants include cassava (*Manihot esculenta* L), avocado and mango. Infection is generally limited to wounded or stress-weakened plants (Britton and Hendrix, 1986). *Lasiodiplodia theobromae* reproduces mainly by asexual macroconidia, with the sexual stage rarely being observed under field conditions (Punithalingam, 1976). The extensive list of synonyms that apply to the species demonstrates the confusion that exists over its taxonomic and phylogenetic status. The standard taxonomic treatment of *L. theobromae* was entirely based on culture characteristics and asexual reproductive structures (e.g. pycnidium, conidiophore, and conidiospores) (Punithalingam, 1976). However, recently, simple sequence repeat (SSR) or microsatellite analysis is widely acknowledged as the method of choice for molecular studies of population genetic structure, relationship, genotype diagnosis and genetic evolution (Cardoso and Wilkinson, 2008).

The two fungal pathogens described above are thought to be introduced into fields on contaminated wood or in infested soils. Once introduced, they have been found to survive for many years in soil or in dead wood. Both fungi invade the plant through wounds and disrupt water and mineral uptake. Infection and disease development are favoured by warm temperatures.

1.1.7.2. Black pod disease

Black pod (Figure 14), caused by several species of *Phytophthora*, is the most widespread disease of cocoa and is found in all cocoa growing regions of the world (Appiah *et al*., 2003; Opoku *et al*., 2005). The disease is responsible for estimated losses of about 450,000 metric tons of global production annually (Langham, 2004). It attacks pods at all stages of their development, and is unique in that there are probably five or more different species of *Phytophthora* that cause pod rot, seedling diseases, and cankers of stems (Appiah *et al*., 2003). *Phytophthora palmivora* is nearly pandemic wherever cocoa is grown, and has a very large host range. *Phytophthora capsici* and *P. citrophthora* may be restricted to the Americas, and *P. heveae* probably is present in South America and Malaysia. On the other hand, *P. megakarya* is present only in West Africa where it attacks only cocoa (Erwin and Ribeiro, 1996; Opoku *et al*., 2005). The disease cycle is difficult to identify because of the several species involved. Wood and Lass (2001) showed that *P. palmivora* does not normally produce sporangia on the surface of diseased pods whereas *P. megakarya* does so abundantly. Sporangia develop when temperatures are 25 to 30 °C with the relative humidity above 80 %, and germinate to produce mycelia, further sporangia or zoospores. *Phytophthora* species are differentiated on the basis of morphological characteristics (Erwin and Ribeiro, 1996) and more recently by molecular-biological means (Appiah *et al*., 2003). The common environmental factors, wind, rain and temperature, influence disease spread, the most important being water.

Figure 14. Black pod disease caused by *Phytophthora* spp.
Photo: USDA, SPCL.

1.1.7.3. Witches' broom disease

Witches' broom (Figure 15) caused by *Crinipellis perniciosa* (Stahel) Singer, is a serious fungal disease of cocoa in Latin America (Pereira, 1999). Indigenous to the Amazon, it is now present in most of the cocoa growing regions in South America and several Caribbean islands. It occurs in Bolivia, Brazil, Colombia, Ecuador, Grenada, Guyana, Panama (on the South American side of the canal), Peru, St. Vincent, Surinam, Tobago, Trinidad, and Venezuela (Purdy and Schmidt, 1996). In 1989, witches' broom was detected for the first time in Bahia state of Brazil. It had invaded the major growing area of Brazil and ravaged production with yields in Bahia decreasing by 60 % from 1990 to 1994. It prevents cocoa pods from being formed and infects mature pods and, therefore, deters many smallholder farmers from growing the crop. *C. perniciosa,* the witches' broom pathogen, is a basidiomycete fungus (Figure 15) believed to have co-evolved with cocoa in its centre of genetic diversity (Allen, 1987; Wheeler and Mepsted, 1988; Purdy and Schmidt, 1996). Witches' broom apparently was observed in the 1700s, but the scientific investigation of this devastating disease began in Surinam in the 1890s (Stahel, 1915) Basidiospores are dispersed by wind, and if they land on dry surfaces, they immediately lose their viability. Moisture must remain on the plant surface for at least 6 hours during which basidiospores germinate, enter cocoa mostly through natural openings, and establish

an infection. Initial infection by *C. perniciosa* occurs in actively growing meristems such as buds, young leaves, flower cushions or pods, causing a characteristic disorganized proliferation of new shoots in the host termed "witches' brooms" (Isaac *et al.*, 1993). Potential crop is lost when clusters of flowers produced on cushions on the main trunk and older branches are infected, thus producing seedless strawberry or carrot-shaped fruits (Pereira, 1999).

Figure 15. Witches' broom (left) caused by *Crinipellis perniciosa* (right = basidiocarp). Photo: USDA, SPCL.

1.1.7.4. Frosty pod disease

Frosty pod (Figure 16) was once a disease of cocoa that occurred only in the coastal hill region of Ecuador (Phillips-Mora, 2003). The fungus that caused the disease was placed in the genus *Monilia*, and was designated *Monilia roreri* (Phillips-Mora, 2003). The disease has carried different names such as Quevedo disease, frosty pod, helada, hielo, moniliasis, podredumbre acuosa, watery pod rot and Monilia pod rot. There is speculation that the pathogen evolved from some unknown source in the rainforest of coastal Ecuador, and spread subsequently into western Colombia.

Phillips-Mora (2003) reviewed the taxonomy of the pathogen of frosty pod and showed that the valid name for the pathogen is *Moniliophthora roreri*, the name selected by Evans (1978). They proposed a close relationship between *C. perniciosa* and *M. roreri* mainly because they are both basidiomycete species and recent molecular-biological studies show that *C. perniciosa* and *M. roreri* are sister taxa that belong in the Marasmiaceae (Aime and Phillips-Mora, 2005).

Figure 16. Frosty Pod caused by *Moniliophthora roreri*
Photo: USDA, SPCL.

1.1.7.5. Vascular streak dieback

Vascular streak dieback caused by the fungus *Oncobasidium theobromae* is a problem in South and Southeast Asia (Wood and Lass, 2001). It attacks the vascular tissues, which transport water and nutrients around the plant. New shoots of infected plants rarely grow more than 20 cm before dying. Vascular streak dieback is distinct from other dieback diseases and was given the name because of the brown streaking of the vascular tissues in diseased plants. The only known host of the pathogen is cocoa (Purdy *et al*., 1998).

1.1.7.6. Cushion galls

This disease is characterized by distortions of flower cushions (Hardy, 1960; Thomas, 1973). Usually the cushion is stripped of its potential to produce normal flowers, or it produces only a few flowers that do not set pods. Hutchins and Siller (1960) studied cushion gall and discussed the symptoms that developed from infection by the fungal pathogen, *Nectria rigidiuscula* (*Fusarium decemcellulare*. The disease is controlled by simple removal and burning of infected trees (Hardy, 1960).

1.1.8. Insects associated with cocoa dieback disease and their management

1.1.8.1. Mirids

Of the more than 1500 species of insects and mites recorded as minor and major pests of cocoa, mirids are the most important (Entwistle, 1972). They are commonly associated with fungal diseases, including tree cankers and pod rots. The association with mirids simply involves creation of an infection point through wound lesions. Cocoa is infested throughout the world by mirids and forty species are known (Entwistle, 1972). They all belong to the subfamily *Bryocorinae*, which is separated into two tribes, Monaloniini or Odoniellini. The Monaloniini include the two genera, *Helopeltis* and *Monalonion*, whilst the Odoniellini include the eight genera *Boxia, Boxiopsis, Bryocoropsis, Distantiella* (Figure 17)*, Odoniella, Platyngomiriodes, Pseudodoniella* and *Sahlbergella*. *Distantiella theobroma* (Dist.), *Sahlbergella singularis* Hagl., *Bryocoropsis laticollis* Schum and *Helopeltis* species are the main mirids occurring on cocoa in West Africa (Squire, 1947), but the most important are *D. theobroma* and *S. singularis*.

Figure 17. Adult female mirids: A = *Distantiella theobroma* B = *Sahlbergella singularis.* Photo: Nick Jessop. Bar = 1 mm.

1.1.8.1.1. Biology and ecology of mirids

Mirids feed on unhardened plant tissue by piercing and sucking plant sap. The components of the mouthparts are the same as the mandibulate type but highly modified to form a system of sharp, elongated piercing organs (stylets) and tubes for drawing up liquid food. The mandibles and maxillae form the needle-like stylets that are protected by a sheath formed from the labium. The whole is called the proboscis or rostrum and measures about 3 mm in length. When the stylets are inserted into the plant the sheath folds outwards. The stylets are grooved and arranged in such a way that saliva is injected down one tube into the tissue while plant juices are sucked up a separate tube. The proboscis extends underneath the body when not in use.

The life history as well as a detailed morphological study of all stages of mirids has been worked out by Cotterell (1926, 1943) and Entwistle (1965). Adult females, with their saw-like ovipositor, insert their eggs into pods and stems. They bury their eggs in plant tissue except for two hair-like filaments that arise from the end of the egg. Cobben (1968) considered the filaments to be bundles of isolated aeropyles, which open individually to the outside in the apical part of the horn. Each horn presumably encloses a single micropyle. The function of the horns is uncertain but Squire (1947) suggested that they draw moisture away from the operculum, thus presumably facilitating respiration. Mating takes place readily in captivity and has been observed to last from one to two hours. The low numerical levels, even at peak mirid populations, suggest a chemical attractant must be involved (Cotterell, 1926). Males of *D. theobroma* were attracted to cages containing females but not to cages containing both sexes. On the other hand, olfactometer studies with this species using laboratory virgin adults, which had never flown, showed no indication of sex attractants (Anon, 1968).

There are five juvenile stages called nymphs. During the last three stages, the wings do not fully develop until the adult stage. The time to egg hatch varies from 13-17 days whilst each of the nymphal stages may last from 3-6 days. Adult females begin to lay eggs about a week after emergence and may lay 30-40 eggs each. There are

insufficient accurate data on the duration of adult longevity due largely to the difficulty of maintaining mirids in captivity. However, most sources suggest that adults of both *D. theobroma* and *S. singularis* live for less than one month. On cut shoots of *Ceiba pentandra* in water, a mean adult longevity of 16.6 days and a mean fecundity of 100 eggs (maximum of 276 eggs) have been recorded for *D. theobroma* in Ghana. With *S. singularis* on stems of cocoa in water, a mean of 57 days and maximum of 179 eggs have been noted (Anon, 1969). If food is available and the climate is favourable, they breed throughout the year. Longevities have been recorded of 7 to 42 days (mean 24.5 days) and 24 to 32 days (mean 28.0 days) for male and female *S. singularis* on the fruit of *Desplatsia dewevrei* under ambient environmental conditions in the insectary. Mirids do not appear to feed during the heat of the day, but rest at the fork and branch unions on the underside of pod stalks, in other protected situations, and are relatively inactive. Patterson (1914) stated that feeding does not begin until 5.30 p.m. unless conditions are dull and wet. If the morning is sunless, they may feed to 10 a.m.

There are generally low population densities of mirids on cocoa but highest numbers occur after the abatement of the major rains. In West Africa, mirids have a distinct population cycle with minimum numbers occurring in the period February to July and maximum numbers from August to January, the position of the maxima and minima varying somewhat from year to year (Entwistle, 1965; Padi and Adu-Acheampong, 2003). It has been suggested that humidity has a direct influence on population changes in West Africa. (Gibbs *et al*., 1968). The preference of *S. singularis* fifth-instar nymphs in laboratory experiments is for high humidity (90 to 95 %) and a temperature (in saturated air) between 18.5 to 23.5 ˚C (Prins, 1965). Choice experiments demonstrated the dominance of the light response over that of humidity, dark situations being chosen irrespective of humidity levels. In view of the susceptibility of mirid nymphs to desiccation, this is a striking result and may well indicate that concealment from predators is of more importance than protection from desiccation (Entwistle, 1972). Its bearing on aggregation of mirids on the tree is obvious but its significance, if any, to inter-tree distribution is uncertain.

Although individual trees may develop high populations, mirids are not particularly numerous on a typical cocoa farm. An average of as few as 112 mirids per ha is

capable of causing very serious damage to cocoa trees (Collingwood, 1971). It is often a source of confusion that such low numbers of mirids should be associated with so much damage. This confusion arises partly because the damage is most impressive after peak numbers have passed and partly because peak numbers are genuinely small compared with many other types of insect pests.

1.1.8.1.2. Symptoms and damage

Mirid feeding lesions on pods and shoots (Figure 18 and 18) provide entry points for spores of pathogenic fungi notably *F. decemcellulare* and *L. theobromae*. The insect itself is not necessarily the source of the pathogens. In Ghana, Crowdy (1947) described a characteristic dieback with three distinct zones in the xylem. The blackish grey dead wood contained both *F. decemcellulare* and *L. theobromae*; the more recently infected zone contained only *F. decemcellulare* while the straw-coloured, water-soaked line separating the infected area from the healthy xylem, rarely contained any fungus. Owen (1956) found that such a zonation was not always evident, and although *F. decemcellulare* was often isolated, it was not always.

Figure 18. Mirid feeding lesions (brown spots) on pods. Photo: Nguessan.

Figure 19. Mirid stem canker on a cocoa tree. Photo: R. A-A.

Canker and dieback are the result when *F. decemcellulare* and *L. theobromae* invade feeding lesions (Cotterell, 1927; Crowdy, 1947). Estimates of the extent of infection of lesions by *F. decemcellulare* in Ghana vary from 80 to 100 % (Hammond, 1957). Fungal infection is not associated only with mirid attack, but may enter through wounds of any kind. From the primary lesion, the fungus invades the xylem, phloem, and medullary rays, with the mycelium confined mainly to lignified tissue. Infected lesions are healed by a growth of callus tissue from the edges, which eventually occludes an area of severely damaged cells in the xylem (Crowdy, 1947). The affected area is recognized by the overlying bark being characteristically rough. The roughness persists for many years and *F. decemcellulare* has been found occluded beneath as much as 7.5 cm of living tissue (Crowdy, 1947). The significance of *F. decemcellulare* lies in the fact that it is a weak parasite, which can be occluded by new growth in healthy trees where it can be dormant for many years only moving into an active phase when the tree becomes weakened. Mirids can kill only young green shoots and such damage is restricted to periods of flush when this type of tissue is present. Young cocoa is particularly susceptible to mirid attack. Thus, mirids make

cocoa difficult to establish and can delay the time for it to come into bearing by several years. In West Africa, there are two predominant patterns of mirid attack known as the 'capsid blast' and 'capsid pockets' (Figure 20).

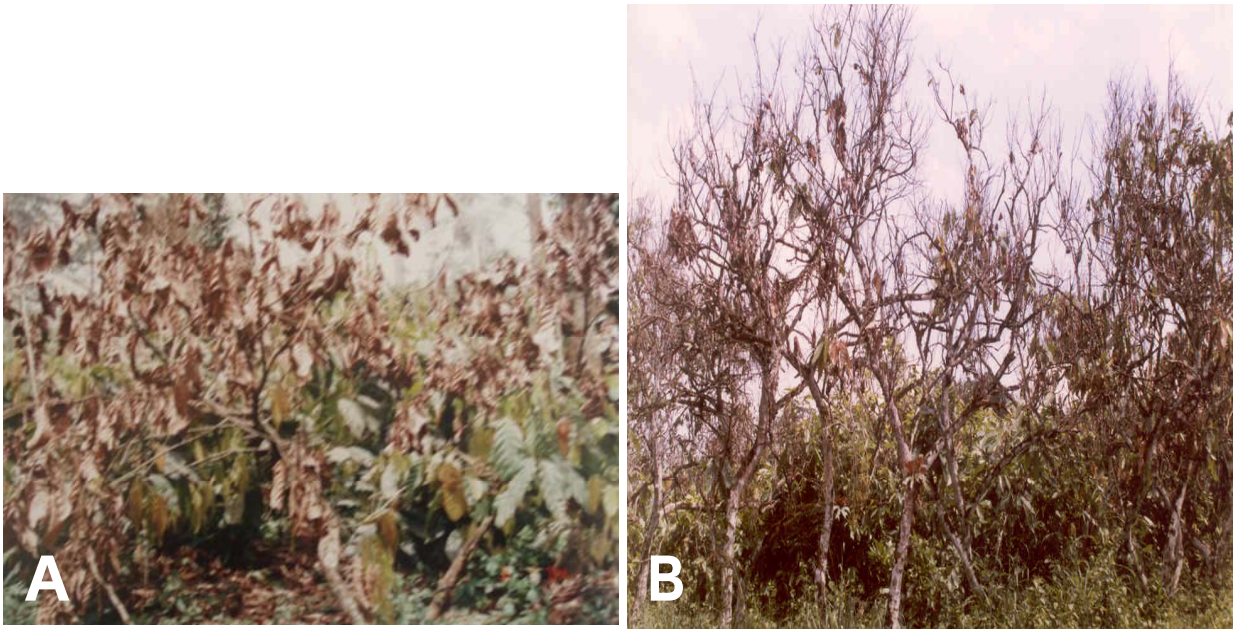

Figure 20. Cocoa trees showing leaf blast and dieback on cocoa farms following fungal infection. Photos: A, R. A-A; B, Nguessan.

'Blast' is recognized by a concentration of attack on fan branches, which results in their death. The dead leaves remain attached to the trees for some time and give a characteristic scorched or blasted appearance. In Ghana, blast is mostly a dry season phenomenon (January-March) and may have very little effect on the tree. In areas where the overhead shade is less dense, degradation of the cocoa canopy itself has consequences that are more serious. 'Pockets' occur when the canopy of trees, up to around one hundred in number, is strongly degraded by intensive feeding on fan branches. If damage persists, trees in the pocket cease to yield and finally die.

Williams (1953) found that 'capsid pockets' were frequently initiated by shade trees falling and breaking the cocoa canopy. Chupon growth was thus encouraged, providing ideal conditions for rapid multiplication of mirids. Stag-headed trees result from the death of the crown following persistent mirid feeding and *F. decemcellulare* dieback on fans and shoots, with mainly dead canopy branches remaining. By total

loss of the canopy remnants, the bare pole stage is reached. The trunk of such trees may have many weak outgrowths.

Damage is not uniformly distributed and severely damaged and healthy trees may occur together. Posnette (1943) found that certain trees appeared to be susceptible to mirid attack while others seemed tolerant, but none of these selections maintained its field behaviour under laboratory conditions.

Mirids on pods feed largely in the parenchymatous husk tissue but the depth of stylet penetration is not known. Cherelles (new pods) may wilt, and pods less than three months old have very little chance of surviving, usually dying from mirid damage or from fungi entering the pods through the lesions (Gerard, 1968). In West Africa, crop losses resulting from this are believed to be negligible because the tree compensates by shedding fewer fruits by cherelle wilt. Well-grown pods seldom seem directly affected. A comparison between ripe pods that had been heavily attacked (more than half the surface blackened by feeding) by *S. singularis* in Nigeria and clean pods revealed no significant differences in dimensions, pod weight, number of beans or weight of peeled beans.

1.1.8.1.3. Host range

Distantiella theobroma and *S. singularis* are native to West Africa. Since cocoa is not indigenous to the region, it is believed that mirids originally fed on native trees (Leston, 1970; Entwistle, 1972), and with the introduction of cocoa, which proved more nutritious, switched from the wild plants to cocoa (Leston, 1970). Entwistle (1972) has listed alternative host plants for the different cocoa mirids.

1.1.8.1.4. Geographic distribution

Entwistle (1972) has given a good account of the distribution of cocoa mirids. *Sahlbergella, Distantiella, Bryocoropsis,* and *Odoniella* occur only in West and Central Africa. In Cote d'Ivoire, *D. theobroma* was said to form about 15 % of the mirid population (Lavabre *et al*., 1963). In Nigeria, it constituted only 3 % of all mirid

collections and is largely restricted to the Western Region (Entwistle, 1964). In the Cameroon, *D. theobroma* has been reported in the proportion of 1:140 (*D. theobroma: S. singularis*) (Lavabre, 1957). In the Central African Republic, it was rare (Boulard, 1967).

1.1.8.1.5. Economic impact of attack

Attempts to estimate losses due to mirids are always complicated by the inadequacy of records and the complexity of losses from other causes such as fungus and virus diseases and physiological dieback (Entwistle, 1965; 1972). However, crop losses in Ghana were estimated at 60,000 to 80,000 tons of dry cocoa (i.e. about 25 %) in 1957.

1.1.8.1.6. Mirid control methods

Biological control

The need for a permanent, cheap, and effective control for cocoa mirids led entomologists to search for natural control agents. Unfortunately, mirids have very few effective natural enemies with only some 20 parasitoid and predator groups being recorded (Entwistle, 1972). There is no known effective mirid predator or parasitoid except casual predation of accidentally exposed nymphs by the ant *Oecophylla longinoda* (Leston, 1970). The predatory ants fall into three, canopy-nesting ants (*Oecophylla*, *Crematogaster* and *Macromischoides*) and two ground or stump nesting ants (*Pheidole* and *Platythyrea*). A salticid spider, *Plexipus paykulli*, has also been recorded, and Reduviid predators include *Rhinocoris obtusus* Beau. and *R. carmelitis* Stal. Parasites include *Euphorus sahlbergellae* Wlk., *Euphorus helopeltides* Ferr. and *Encyrtus cotterelli* Watson (Hymenoptera: Encyrtidae). A number of these were once eagerly sought in Ghana as biological control agents (Lodos, 1968; Entwistle, 1972; King, 1971), but the benefits were doubtful even when these predators and parasites became established. They are therefore no specific natural enemies of mirids.

Chemical control of mirids in Ghana

Cocoa dieback management in W. Africa largely involves the use of conventional insecticides against mirids (Padi and Owusu, 2001), as a great deal of the research on

mirid damage control focuses on control of the insect, rather than on any associated pathogen. No methods have been developed over the past 50 years for control of *F. decemcellulare* or *Lasiodiplodia* since Crowdy (1947), and Owen (1956) measured pathogenicity of the fungus in W. Africa. Long-residual insecticides such as dieldrin, aldrin and γ-BHC (Entwistle, 1972) were used in the middle of the last century. Although two to three applications could control mirids for over a year in the early days of their use, the combination of resistance build-up and governmental restrictions on the use of persistent chlorinated hydrocarbons led to a changeover to carbamates, newer nitroguanidines, and pyrethroids, such as promecarb, imidacloprid and bifenthrin, respectively (Padi and Owusu, 2001). These, however, also pose problems as chemical control is not an environmentally friendly option and consumers are conscious about residues. Input costs are increasing, which are not conducive to increasing profitability. Non-chemical mirid control measures as part of an integrated pest management strategy are therefore essential for the success of the crop. Recent studies on the use of sex pheromones, botanical pesticides and myco-insecticides offer hope for the future (Padi *et al*., 2001).

1.1.9. Prospects for the use of host plant resistance for the control of mirid-related dieback of cocoa

West Africa produces about 70 % of the world's cocoa but the region suffers from severe disease and pest attack notably by *Phytophthora* species and mirids. Mirid related dieback is estimated to cause annual economic losses of several million dollars in Ghana alone, and the higher losses are slowing farmers' interest in cultivating the crop. Further, as such losses of produce from disease are serious enough to warrant the application of pesticides, the possibility of contamination of the environment. In 2007, the government of Ghana spent ¢ 479.91 billion (USD 51 million) for the year's Cocoa Diseases and Pests Control (CODAPEC) programme (The Ghanaian Observer, Wednesday, June 06 2007). Breeding plants for resistance to pathogens is another form of biological pest control where researchers look for genetic traits that reduce a plant's susceptibility to attack or injury by its pathogens. Disease resistance is a fundamental component in IPM and sustainable agriculture. The idea is not new and a

number of examples have been described including leaf blights in maize, late blight in potatoes, rice blast, stripe rust of wheat, rust in groundnut and powdery mildew in barley (Jacobs and Parlevliet, 1993).

Following the chemical revolution of the early 20th Century, research into alternative control measures for plant diseases, prompted by environmental concerns, has shifted from pest control utilising chemicals towards more sustainable methods including biological controls, genetic resistance, integrated pest management (IPM) and natural products (Cook and Baker, 1996). Current interest in host plant resistance in cocoa stems from environmental and socioeconomic policy concerns including global climate change, local air and water quality, pesticide residues and security of cocoa supply. Resistant cocoa germplasm will provide good yields at minimal costs with no adverse effects on non-target organisms and zero pesticide residues. Further, the environment with greater biodiversity of both plants and animals will likely provide ecosystems with greater resiliency. In that case, the cocoa supplies system will be more secure.

1.2. Overall thesis objective:

The overall objective of this thesis was to evaluate responses of some cocoa genotypes to infection in terms of dieback disease and necrotic canker caused by *F. decemcellulare* and other pathogenic fungi. To be able to engage the problem, detailed studies of the different pathogens and their genetic diversity, along with assessments of their pathogenicity was needed. Specifically, an understanding of the following questions was needed: How many pathogenic species are involved with dieback and canker? Are the different species equally pathogenic, and does aggressiveness change allowing a particular species to overcome others at a particular circumstance? How do environmental conditions affect the pathogenicity of the isolates? What will be a suitable and reliable method of artificial inoculation? Finally, to understand the reaction of different cocoa genotypes to infection and to select resistant plants, knowledge of some resistance variables was needed, including incubation period, rate and direction of fungal spread within the plant, time course of development of necrotic lesions and dieback severity. Several million Ghanaian cedis are spent annually on controlling dieback disease through the national mass-cocoa

spraying programme (against mirids), and therefore, the selection of resistance clones is critically needed now more than ever.

1.2.1. Thesis outline

The study was aimed at sustaining West African cocoa breeding programmes with respect to resistance to *Fusarium decemcellulare* and *Lasiodiplodia theobromae*, the causal agents of dieback disease with the view to minimising pesticide use. Chapter 1 is a review of the general literature accumulated over the past 100 years on fungal diseases of cocoa, dieback disease, *F. decemcellulare*, *L. theobromae* and mirid insects. It was necessary to review the present knowledge about *F. decemcellulare* and *L. theobromae*-cocoa interaction to gain insight into the problems of breeding for dieback disease control. The review covers all aspects pertaining to the cocoa ecology and production, *F. decemcellulare* and *L. theobromae* taxonomy and pathogenicity. Chapter 2 reports work in the laboratory and the greenhouse on the morphological variability and pathogenicity of isolates obtained from diseased cocoa trees. Chapter 3 deals with the genetic diversity within and between the different isolates. Chapter 4 explains the reaction of a range of cocoa clonal genotypes to infection by *F. decemcellulare* and *L. theobromae* and shows the results of laboratory and greenhouse methods and their use in identifying resistant clones. Inoculation techniques initially developed in late 1940s and '50s were improved and used to screen cocoa in greenhouse tests. From the laboratory and greenhouse screenings, which were carried out with some *F. decemcellulare* and *L. theobromae* isolates, a few resistant clones were identified, including CATIE 1000, T85/799, and MXC 76. The thesis concludes with a general discussion (Chapter 5). In this chapter the results from the previous chapters are reviewed, and their implications for cocoa dieback research and practical breeding for resistance to *F. decemcellulare* and *L. theobromae* are discussed.

2. Chapter 2

2.1. Characterization and pathogenicity of isolates of *Fusarium* and *Lasiodiplodia* species from dieback lesions of cocoa in Ghana

2.2. Introduction

Fungal pathogens are very common on plants (Agrios, 2005) and they have assumed greater importance in both developed and developing countries particularly due to the increased intensity of agriculture and the introduction of highly improved planting materials. The hot-humid tropics and subtropics offer very conducive conditions for fungal disease spread. A group of ascomycete fungi that require wounds to infect, cause dieback of cocoa as mentioned in the preceding chapter. The technical presentation of this disease, though very typical of fungal infection, is often confused with insect disorders mainly due to the relationship between mirid feeding punctures and disease occurrence. This probably led to mismanagement of the disease in the past through uncontrolled application of broad-spectrum insecticides leading to resistance development in mirids (Dunn, 1963). This chapter presents results on morphological characterisation and pathogenicity of the causal pathogens of dieback, and the effects of drought and relative humidity on disease development in the greenhouse.

By analogy with other disease complexes, variation in pathogenicity among the isolates could result from genetic differences between isolates of a single species or may stem from the presence of multiple species. The objectives of this chapter were to address the role and identity of the different fungi associated with dieback disease of cocoa in Ghana. Information needed includes (a) whether cocoa is infected by a fairly uniform and stable population of fungi, (b) whether this population can, under natural conditions, cause dieback disease of cocoa, (c) whether there are other fungi present which have not been identified because the cocoa that is grown is not susceptible, (d)

whether the isolates affecting cocoa in West Africa are the same as those attacking it in other regions (e) whether the fungal population can be determined as distinct on some simple independent basis such as colony morphology, temperature requirement, pathogenicity or growth rate? Answers to these questions would enable more realistic conclusion to be drawn on questions of relationship and biology of the pathogen populations.

2.3. Materials and methods

2.3.1. Comparative isolation rates of different fungi from infected cocoa stems on different culture media

Stems of cocoa trees measuring about 15 cm long from trees showing dieback symptoms were obtained from cocoa farms in the Eastern Region of Ghana. A modified procedure of Owen (1956) was used to isolate *Fusarium* and *Lasiodiplodia theobromae* from the infected cocoa stems. Four different media formulations were screened in initial trials, namely:

a) Potato dextrose agar (PDA): as per manufacturer's instructions

PDA (Merck, Germany)		39 g
Distilled water to		1 litre

b) Potato carrot agar (PCA)

Grated potato		20 g
Grated carrot		20 g
Purified agar (OXOID®)	20g	
Tap water to		1 litre

c) Carnation leaf agar (CLA)

Fresh carnation leaves		20 g
Purified agar (OXOID®)	20g	
Tap water to		1 litre

d) Cocoa pod husk agar (CPHA)

Dry (chopped) cocoa pod husk	20 g	
Purified agar (OXOID®)	20g	
Tap water to		1 litre

All media preparations were sterilised at 121 ˚C for 15 min. The sterilised media were mixed thoroughly and poured into Petri dishes (20 ml per 9-cm plate). The infected stems were cut and sliced to smaller pieces, cleaned externally with 70 % ethanol for about 10 seconds followed by 0.5 % sodium hypochlorite for 1min. They were then rinsed in sterile de-ionized water and blotted dry on sterile paper towel prior to being placed in the Petri plates. The tissues were incubated at 30.0 ± 2.0 ºC with 12 hours of light and 12 hours of darkness for 14 days to isolate the pathogens and other endophytic organisms. Potato dextrose agar was taken as the standard medium for primary isolation and the others were compared with it. The efficiency of the four media used for isolation of the fungi was compared based on the extent of growth of the colonies on the plates.

The geographic origin of the isolates is given in Table 4 (page 63). Isolates were identified provisionally, using standard mycological keys, and grouped into genera. One hundred and seventeen (117) Ghanaian isolates belonging to either *Fusarium* spp. (75 isolates) or *L. theobromae* (36 isolates) (Table 4) were obtained from the diseased stems. Four *Fusarium* isolates included in the samples evaluated were received as PDA cultures collected from cocoa in the Sefwi-Boako area of the Western Region of Ghana. These were stored on sterile PDA at 4 ºC.

2.3.2. Pure cultures from single spore isolates

Each of the 117 isolates was treated as a separate individual. After confirmation of their vegetative structures, conidia were transferred to PDA slants and incubated as before. To obtain pure cultures from single spores, concentrations of spore suspensions were adjusted to approximately 10 spores per microlitre. A permanent marker was used to draw circles (about 3 mm diameter) on the bottom of each PDA plate (9 cm diameter). A 1.0 μl drop of the spore suspension was placed on the surface of the PDA above each circle. After incubation at 30 °C for 24 hours, each circle was inspected under a light microscope at × 100 magnification from the bottom of the plate. Those circles containing single germinating spores were marked and the spores in them transferred to fresh PDA plates. All isolates were stored as mycelium on PDA slants at 4 °C.

2.3.3. Morphological Studies

2.3.3.1. Colony and conidial morphology

A total of 117 isolates were obtained from infected cocoa stems from the Eastern (Tafo) and Western (Sefwi-Boako) Regions of Ghana. Thirty-six were identified as *L. theobromae* from morphological features while the remainder produced *Fusarium*-type spores (Leslie and Summerell, 2006). Two reference isolates of *F. decemcellulare* (IMI 380504 and IMI 361352) and one *L. theobromae* isolate (IMI 333797) were obtained from the CABI Fungal Genetic Resource Collection. Dr Gary Samuels of the United States Department of Agriculture (USDA) also kindly provided two-reference *Fusarium decemcellulare* isolates (GJS 03-81 and GJS 01-170).

A mycelial block about 5 × 5 × 5 mm was cut from the advancing margin of each single spore culture and placed in the centre of a Petri dish filled with PDA. Using the key of Nobles (1958), the macroscopic characters (colour, sector, border, and texture) of each colony were recorded at 24-hour interval until the colonies had reached the rim of the plate. Measurements of conidia from 20 to 25 observations per isolate were made with a light microscope at × 400 magnification. A count of spores was done in a haemocytometer (Fuchs-Rosenthal). For most measurements, 10 haemocytometer squares (0.25 × 0.25 mm) were counted per slide.

2.3.3.2. Growth rate measurement of colonies

To determine the linear growth rate of isolates, one 12-mm diameter plug of PDA with mycelium was removed from each of 7-day-old cultures using a sterile cork borer, and placed at the centre of a PDA plate, five replicates being made for each isolate. The five sets of each isolate were grown upside down in Petri plates to minimise moisture accumulation on the agar surface. The plates were incubated in darkness at temperatures ranging from 10 to 35 ˚C at 5 ˚C intervals. Mycelial growth measurements were recorded at 24-hour interval until one of the colonies had reached the border of the plate.

2.3.3.3. The longevity of conidia

The longevity of the four reference isolates of *F. decemcellulare* and four isolates each of *Fusarium* spp. and *L. theobromae* were investigated in the laboratory. Colonies of the different isolates on PDA were kept in the incubator at 25 ˚C under cool white light and the viability of 100 macroconidia from each plate was examined by simple germination tests on microscope slides once every month.

2.3.3.4. Competition in culture

Isolates of *F. decemcellulare*, *Fusarium* spp. and *L. theobromae* were grown on PDA plates at 30 °C for two weeks. Plugs (5 mm diam.) were aseptically removed from actively growing colonies and transferred, singly or two or three fungi together, to plates of PDA. The plugs were placed at the centre of the plates when the fungi were singly grown, or at the corners of a triangle, 2 cm from the centre, when they were grown together. The experiment was run with 10 replicates. All plates were sealed with Parafilm and incubated at 30 °C in the dark. Growth of the colonies was measured daily for one week.

2.3.4. Greenhouse pathogenicity tests

Three trials were conducted during 2006 and 2007 to test the pathogenicity of *Fusarium* species (including four reference *F. decemcellulare* isolates) and *Lasiodiplodia* isolates collected from field-infected cocoa trees from Ghana. Unless otherwise stated, observations of treated plants ended after 12 weeks.

2.3.4.1. Plant materials and growth conditions

Pods of the Amelonado cocoa variety were obtained from research plots at the Cocoa Research Institute of Ghana (CRIG). The seeds were washed under tap water, surface-sterilized in 0.5 % sodium hypochlorite for one minute, rinsed in distilled water for five minutes, and air-dried for thirty minutes. The seeds were planted 20 mm deep into 50 cm × 50 cm plastic trays filled with autoclaved soil consisting of a 2: 2: 1 mixture of multi-purpose compost, loam, and sharp sand. When the plants were at the true leaf stage (i.e. after 14 days), two seedlings were transplanted into 18 cm × 12 cm

plastic pots containing sterilised soil, and after 28 days, these were thinned to one plant per pot (Figure 21). Growing temperatures in the greenhouse ranged from 27 to 45 during the day and 20 to 22 °C at night.

Figure 21. Amelonado cocoa seedlings growing in the greenhouse. Photo: R. A-A.

In the summer of 2006, the greenhouse compartment became overheated and it was essential to provide additional shading. This was achieved by transferring plants underneath the benches with large trays arranged on top of the benches. The plants were watered as necessary and, when needed, fertilized with 'GrowMore', a source of nitrogen, phosphorus and potassium. Pod husks were autoclaved before disposal. All seedlings were maintained in the greenhouse until fungal pathogenicity studies began. At the beginning of the study, all plants were two months old and had an average height of 32 ± 6 cm. Thus, Amelonado seedlings were used both for the pathogenicity tests as well as the rootstock for the cocoa genotypes discussed in Chapter 4, as this variety is known to be susceptible to both *F. decemcellulare* and *L. theobromae* canker under natural conditions (Owen, 1956).

In order to determine pathogenicity, four plants of Amelonado seedlings were tested for each isolate from diseased cocoa trees from Ghana and the five reference isolates from CABI and USDA. The plants were inoculated at two-month old using two alternative methods (i. scalpel wound-mycelium plug and ii. needle-wound conidial suspension inoculation). The fungal cultures (*Fusarium* species, *F. decemcellulare* and *L. theobromae*) were maintained for up to four months on PDA slants in a refrigerator at 4 ˚C before being transferred to new PDA slants. Fourteen days prior to inoculation, cultures were transferred from slant tubes to PDA plates. After inoculation and during the subsequent three days, potted plants were kept in a dew chamber at 27 ˚C. The experiment was conducted under high soil moisture and the surrounding air humidity in the greenhouse was maintained at 50 to 90 % by daily spraying with water. Isolates were considered non-pathogenic if no wilt symptoms occurred after 45 days in all plants inoculated with that isolate.

2.3.4.2. Agar plug inoculation

A small wound (5 mm^2) was made on the stem of each seedling after surface sterilization with 70 % ethanol, by removing the bark and exposing the cambium. Mycelial plugs of similar size in mm overgrown with the test fungi were placed into each wound with the mycelium facing the cambium. Inoculated wounds were covered with Parafilm tape to prevent desiccation of the inoculum. For control inoculations, sterile PDA plugs were inserted into wounds on the stems of four plants.

2.3.4.3. Spore inoculation

Macroconidia from isolates (excluding those that did not produce visible symptoms in the agar plug inoculation described above) were harvested from ten-day old PDA cultures grown at 25 °C by flooding them with sterile distilled water containing 0.025% Tween 20. Pycnidia of *L. theobromae* were crushed into the water. The resulting aqueous suspension of macroconidia was vortexed and serial dilutions made to the following concentrations, 1×10^3, 1×10^4 1×10^5 and 1×10^6 conidia per ml. The spore concentrations were determined using a Fuchs-Rosenthal haemocytometer

(depth 0.2 mm, $^{1}/16$ mm^2). These concentrations, with the addition of a sterile distilled water control, were inoculated into Amelonado seedlings (four plants per concentration) by puncturing the bark of the main stem with a needle and 10-µl inoculum injected with a syringe and needle into the small puncture of each seedling. The sites of inoculation were covered with strips of sterile moist paper towels or cotton wool and Parafilm to maintain high humidity. After inoculation, the plants were kept in a dew chamber for three days and were then transferred to the greenhouse at 23 to 27 ˚C and 60 to 90 % relative humidity for disease development. The minimum effective dose was defined as the lowest conidial concentration that produced dieback in 90 % of the plants relative to the control.

In both the agar plug and spore inoculation tests, the treated seedlings were kept physically separated to avoid cross contamination. They were examined weekly for 12 weeks to reveal differences in the development of dieback and internal stem necrosis. First, a diagrammatic scale consisting of four scores based on stem and crown death was used (Figure 22). In this scale, score 1 = plants with no visual symptoms, 3 = slight distortion of apical leaves and visible browning on leaves, or those that show signs of recovery 6 = isolated patches of dead leaves appearing half-wilted, half-green, and score 9 = dead plant i.e. no green tissue left. Secondly, the stems were sliced and the visible internal dark-brown lesions most likely resulting from infection were observed and measured under magnification. Thirdly, the length of the incubation period was recorded. The experiment was set up in a randomized complete block design with four replicates and was repeated once. Isolates that caused neither wilt symptoms nor vascular necrosis were considered non-pathogenic and excluded from further tests.

Pictorial guide	(1-9) score	Damage to foliage and shoot
	1	No infection observable
	3	Wilting of lowest leaves
	6	Foliage appears ½ wilted ½ green
	9	Leaves/stem dead - no green tissue left

Figure 22. A pictorial guide used for assessing the level of infection of inoculated plants.

2.3.4.4. Effect of relative humidity and soil moisture on disease development in Amelonado seedlings

The effects of relative humidity (r.h.) and soil moisture on disease development were studied using four isolates, AC371, AC845, IMI380505 and IMI361352. Two month-old Amelonado cocoa seedlings were inoculated with 10 µl of spore suspension per plant (at a concentration of 1×10^6 conidia per ml). The experiment was conducted in two adjacent greenhouse compartments (8.5 × 7.5 m) designated as high and low r.h. environments. Both environments had two groups of plants. One group received high soil moisture, the other low soil moisture. The greenhouse was misted each morning with a hose misting system and pots watered every other day with tap water. In the set of plants maintained at low soil moisture, water deficit was achieved by withholding water two weeks prior to, and two weeks after inoculation and then supplementary water provided to maintain the plants at approximately the same level of stress. Each treatment was replicated five times. An Oregon scientific thermo hygrometer MPT 1340 was installed to monitor temperature and humidity conditions in each compartment. Temperature was controlled by an automatic venting system. The incubation period and the number of plants showing signs of disease were recorded at the end of the trial (i.e. 4 weeks after inoculation).

2.3.4.5. Effect of route of entry, inoculum type and plant part on disease development in Amelonado seedlings

The principles underlying these experiments were similar to those discussed above for the effects of relative humidity and water deficit but the methods used differed. The top three fully expanded leaves were inoculated with 10 µl of macroconidial suspension (1×10^6 per ml in sterile distilled water) of four isolates, AC371, AC845, IMI380505 and IMI361352 using a micropipette. Leaves were inoculated by applying the spore suspension or agar to needle wounds on the under surface of each leaf. For agar plug inoculation, the petiole or pulvinus was wounded with a needle, and

inoculated with a small block of fungal mycelium. As a control, leaves were treated with sterile distilled water or sterile agar only. As described above, the plants were placed inside a dew chamber for three days to maintain a saturated environment favourable for infection then subsequently returned to the greenhouse. Pathogenicity to leaf petioles and pulvini, under the same conditions as those used for the leaf inoculation, was also studied.

2.3.4.6. Inoculation on stem and leaf discs

A laboratory *in-vitro* test involved freehand stem sections (2 mm thick, 3-4 mm diameter) and detached leaf discs (12 mm diameter discs punched out with a sterile cork borer) (Figure 23). The stem sections and leaf discs were washed in 70 % ethanol for 5 to 10 seconds, followed by 0.5 % sodium hypochlorite for 1 minute, washed three times in distilled water, blotted dry, and transferred (lower leaf surface up) to a special transparent cover box (28.5 × 15.5 × 8.5 cm) lined with moist tissue paper to maintain a near-saturated atmosphere. Spore suspensions were prepared separately for the four isolates in sterile distilled water in a test tube as described above, agitated for one minute with a Vortex mixer, and the concentration for each isolate adjusted to 1×10^6 macroconidia per ml. The stem sections and leaf discs were inoculated with 5 µl of spore suspension using an Eppendorf micropipette. As a control, stem sections and leaf discs were treated with 5 µl of sterile distilled water. Inoculated tissues were incubated under high humidity at room temperature and observed for the growth of mycelium. For light microscopy, leaf discs were cleared with a saturated solution of chloral hydrate and stained with cotton blue in lactophenol.

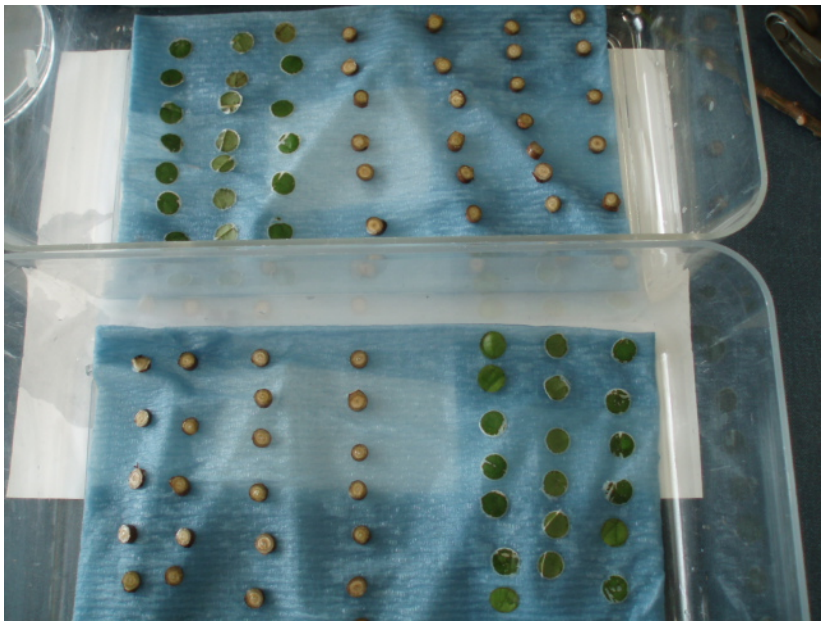

Figure 23. Set-up of in-vitro leaf discs and stem section inoculation using spore suspension.

2.3.4.7. Re-isolation of pathogens from inoculated plants

Isolations were made from cankers on samples of seedlings to determine whether the disease had been caused by the inoculated fungi. This was done by cutting infected plant tissue both above and below the wound, into a number of stem segments, and surface sterilizing them in 70 % ethanol for 5 to 10 seconds, followed by 0.5 % sodium hypochlorite for one minute, and washing three times in distilled water. The plant cuttings were plated on PDA and the plates were checked at intervals.

2.3.4.8. Data analysis

Whenever possible, ANOVA, regression and Kruskal-Wallis non-parametric tests were applied to the data, and Least Significant Difference test ($P \leq 0.05$) used to determine the differences between isolates. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS), Minitab, Microsoft Excel and R.

2.4. Results

2.4.1. Pathogen isolation and description

A total of 117 specimens from the infected stems (including the four PDA cultures from Ghana) were grown for two weeks on PDA and the different colonies were subcultured for purity. Microscopic examination showed that 81 isolates were *Fusarium* spp. while 36 were *L. theobromae* based on colony and conidial morphology of the reference isolates (Table 4). Figure 24 illustrates the relative frequencies with which the main fungi associated with dieback disease were recovered from diseased cocoa trees from Ghana in 2005. *Fusarium* spp. was the primary group from the diseased trees followed by *L. theobromae.* *Lasiodiplodia theobromae* was the single most isolated species from the canker lesions found in cocoa from the Eastern Region of Ghana. This fungus is known to cause cankers and dieback on a wide range of woody hosts, and although not closely related to *Fusarium*, it produced cankers and dieback on cocoa visually indistinguishable from those produced by the *Fusarium* isolates. In general, *Fusarium* spp. were found in plants that were also infected by *L. theobromae,* but they were significantly more abundant than *L. theobromae.* No cultures corresponding to reference isolates of *F. decemcellulare* were found. This study provides the first evidence that species of *Fusarium* other than *F. decemcellulare* also cause dieback disease in cocoa seedlings. As shown in Table 5 below, all the isolates grew on all the four media tested, but they grew best on PDA followed by PCA medium. Cocoa-pod-husk agar and CLA generated lower numbers of fungal colonies which grew more slowly.

When PDA plates were subcultured via spores, both *F. decemcellulare* and *Fusarium* spp. retained their pinkish-red to creamy-white characters but they lost a great deal of their colour after successive subculturing on PDA by mycelial transfer. The associated loss of pinkish or creamy coloration over generations varied among the isolates, but examination showed that conidial cultures were consistently more uniform based on their colour than when the colonies were developed from mass agar transfers. Usually the colours of the latter changed from creamy-white to white.

Table 4. Characteristics of *Fusarium decemcellulare*, *Fusarium* spp. and *Lasiodiplodia theobromae* isolates on PDA

Fungus	Isolate	Geographic origin	Morphological characteristics		
			Colony colour	*Conidium length [a] ± s.e.	*Conidium width [a] ± s.e.
F. decemcellulare	IMI 380504	Ghana	Pinkish	66.00 ± 0.086	9.10 ± 0.044
	IMI 361352	Malaysia	Pinkish	66.00 ± 0.106	9.10 ± 0.057
	GJS 03-81	Brazil	Pinkish	66.22 ± 0.065	9.25 ± 0.055
	GJS 01-170	Cameroon	Pinkish	65.85 ± 0.117	9.12 ± 0.048
Fusarium species	AC 037	Eastern, Ghana	Creamy-white	39.77 ± 0.074	3.17 ± 0.055
	AC 110	Eastern, Ghana	Creamy-white	37.90 ± 0.044	3.42 ± 0.053
	AC 120	Eastern, Ghana	Creamy-white	37.97 ± 0.065	3.30 ± 0.067
	AC 174	Eastern, Ghana	Creamy-white	37.85 ± 0.062	3.36 ± 0.062
	AC 197	Eastern, Ghana	Creamy-white	38.92 ± 0.039	3.20 ± 0.057
	AC 206	Eastern, Ghana	Creamy-white	36.57 ± 0.101	3.47 ± 0.048
	AC 229	Eastern, Ghana	Creamy-white	38.45 ± 0.092	3.22 ± 0.055
	AC 255	Eastern, Ghana	Creamy-white	37.80 ± 0.074	3.50 ± 0.044
	AC 276	Eastern, Ghana	Creamy-white	35.87 ± 0.048	3.35 ± 0.051
	AC 284	Eastern, Ghana	Creamy-white	36.77 ± 0.074	3.43 ± 0.053
	AC 328	Eastern, Ghana	Creamy-white	38.90 ± 0.044	3.27 ± 0.048
	AC 330	Eastern, Ghana	Creamy-white	34.80 ± 0.074	3.50 ± 0.044
	AC 402	Eastern, Ghana	Creamy-white	39.90 ± 0.044	3.02 ± 0.039
	AC 451	Eastern, Ghana	Creamy-white	38.80 ± 0.074	3.41 ± 0.044
	AC 593	Eastern, Ghana	Creamy-white	38.85 ± 0.071	3.32 ± 0.039
	AC 638	Eastern, Ghana	Creamy-white	37.97 ± 0.042	3.35 ± 0.033
	AC 656	Eastern, Ghana	Creamy-white	35.92 ± 0.039	3.22 ± 0.039
	AC 705	Eastern, Ghana	Creamy-white	38.85 ± 0.062	3.40 ± 0.044
	AC 773	Eastern, Ghana	Creamy-white	38.87 ± 0.059	3.42 ± 0.039
	AC 914	Eastern, Ghana	Creamy-white	37.82 ± 0.073	3.31 ± 0.044
	AC 317	Eastern, Ghana	Creamy-white	41.92 ± 0.063	3.65 ± 0.033
	AC 511	Eastern, Ghana	Creamy-white	41.97 ± 0.042	3.47 ± 0.042
	AC 787	Eastern, Ghana	Creamy-white	41.90 ± 0.057	3.42 ± 0.039
	AC 806	Eastern, Ghana	Creamy-white	41.87 ± 0.059	3.50 ± 0.044
	AC 031	Western, Ghana	Creamy-white	42.92 ± 0.063	3.15 ± 0.048
	AC 392	Western, Ghana	Creamy-white	41.85 ± 0.071	3.32 ± 0.039
	AC 748	Western, Ghana	Creamy-white	42.02 ± 0.065	3.30 ± 0.060
	AC 767	Western, Ghana	Creamy-white	43.97 ± 0.065	3.02 ± 0.065
	AC 076	Eastern, Ghana	Pinkish-white	61.80 ± 0.074	4.67 ± 0.048
	AC 211	Eastern, Ghana	Pinkish-white	59.95 ± 0.078	4.02 ± 0.065
	AC 219	Eastern, Ghana	Pinkish-white	58.05 ± 0.069	3.97 ± 0.042
	AC 361	Eastern, Ghana	Pinkish-white	62.02 ± 0.065	4.25 ± 0.033
	AC 551	Eastern, Ghana	Pinkish-white	60.95 ± 0.060	4.95 ± 0.033
	AC 580	Eastern, Ghana	Pinkish-white	60.87 ± 0.069	5.03 ± 0.033
	AC 739	Eastern, Ghana	Pinkish-white	58.85 ± 0.062	4.90 ± 0.044
	AC 768	Eastern, Ghana	Pinkish-white	61.84 ± 0.078	4.55 ± 0.048
	AC 995	Eastern, Ghana	Pinkish-white	60.87 ± 0.059	4.50 ± 0.044
Lasiodiplodia species	IMI 333797	Nigeria	Grey to black	22.44 ± 0.133	12.25 ± 0.119
	AC 008	Eastern, Ghana	Grey to black	22.02 ± 0.065	12.10 ± 0.156
	AC 036	Eastern, Ghana	Grey to black	21.90 ± 0.057	11.90 ± 0.057
	AC 064	Eastern, Ghana	Grey to black	21.97 ± 0.082	12.02 ± 0.042
	AC 068	Eastern, Ghana	Grey to black	21.92 ± 0.095	11.97 ± 0.065
	AC 280	Eastern, Ghana	Grey to black	22.05 ± 0.048	12.00 ± 0.061
	AC 318	Eastern, Ghana	Grey to black	22.10 ± 0.067	12.07 ± 0.063
	AC 322	Eastern, Ghana	Grey to black	22.07 ± 0.053	12.05 ± 0.048
	AC 329	Eastern, Ghana	Grey to black	22.05 ± 0.060	12.02 ± 0.055
	AC 360	Eastern, Ghana	Grey to black	22.00 ± 0.100	12.02 ± 0.065
	AC 371	Eastern, Ghana	Grey to black	22.10 ± 0.067	12.10 ± 0.067
	AC 375	Eastern, Ghana	Grey to black	22.05 ± 0.085	12.10 ± 0.057
	AC 383	Eastern, Ghana	Grey to black	21.95 ± 0.069	11.97 ± 0.065
	AC 407	Eastern, Ghana	Grey to black	22.12 ± 0.085	12.07 ± 0.053
	AC 450	Eastern, Ghana	Grey to black	22.05 ± 0.048	12.05 ± 0.048
	AC 492	Eastern, Ghana	Grey to black	22.12 ± 0.059	12.10 ± 0.057
	AC 536	Eastern, Ghana	Grey to black	22.07 ± 0.053	12.05 ± 0.048
	AC 564	Eastern, Ghana	Grey to black	22.17 ± 0.063	12.10 ± 0.044
	AC 568	Eastern, Ghana	Grey to black	22.12 ± 0.063	12.15 ± 0.062
	AC 581	Eastern, Ghana	Grey to black	22.05 ± 0.048	12.02 ± 0.042
	AC 640	Eastern, Ghana	Grey to black	22.17 ± 0.063	12.17 ± 0.063
	AC 644	Eastern, Ghana	Grey to black	22.05 ± 0.048	12.00 ± 0.061
	AC 680	Eastern, Ghana	Grey to black	22.02 ± 0.065	12.00 ± 0.070
	AC 742	Eastern, Ghana	Grey to black	22.20 ± 0.054	12.20 ± 0.054
	AC 810	Eastern, Ghana	Grey to black	22.27 ± 0.074	12.20 ± 0.074
	AC 845	Eastern, Ghana	Grey to black	22.07 ± 0.053	12.07 ± 0.053
	AC 857	Eastern, Ghana	Grey to black	22.07 ± 0.039	12.05 ± 0.048
	AC 972	Eastern, Ghana	Grey to black	22.15 ± 0.062	12.15 ± 0.062
	AC 220	Eastern, Ghana	Grey to black	22.17 ± 0.053	12.15 ± 0.062
	AC 420	Eastern, Ghana	Grey to black	22.27 ± 0.074	12.22 ± 0.074
	AC 456	Eastern, Ghana	Grey to black	22.17 ± 0.063	12.15 ± 0.071
	AC 487	Eastern, Ghana	Grey to black	22.10 ± 0.057	12.02 ± 0.055
	AC 496	Eastern, Ghana	Grey to black	22.12 ± 0.048	12.07 ± 0.063
	AC523	Eastern, Ghana	Grey to black	22.05 ± 0.048	12.02 ± 0.055
	AC 639	Eastern, Ghana	Grey to black	22.15 ± 0.062	12.12 ± 0.069
	AC 673	Eastern, Ghana	Grey to black	22.20 ± 0.065	12.00 ± 0.061
	AC 718	Eastern, Ghana	Grey to black	22.20 ± 0.054	12.05 ± 0.069

[a] All measurements are in µm; values are means of 20 macroconidial* measurements.

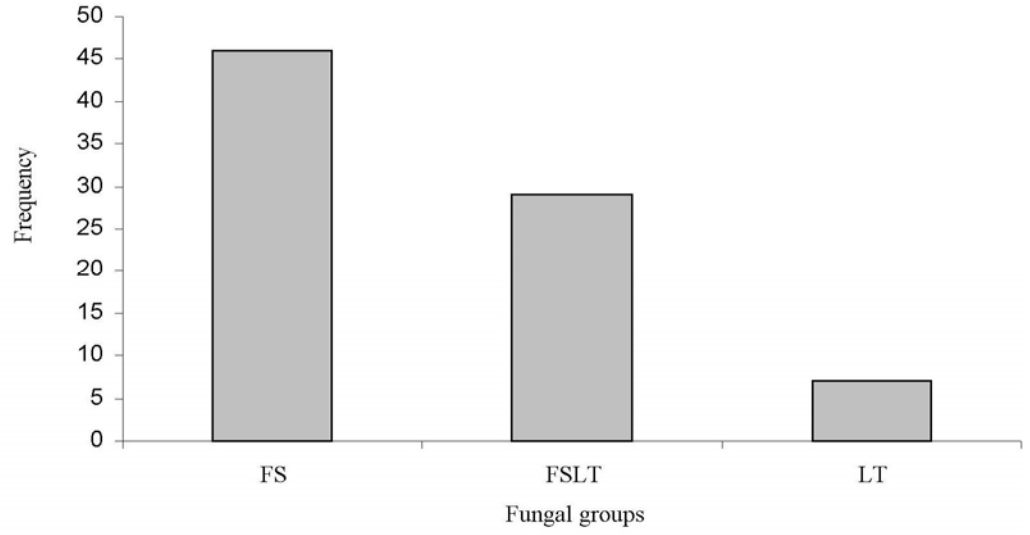

Figure 24. The frequency distribution (number of times isolated) of the different fungi in infected wood. FS = occurrence of *Fusarium* species; LT = occurrence of *Lasiodiplodia* species; FSLT = occurrence of both fungi in the same wood (n = 82).

The colonies of the four *F. decemcellulare* accessions and those of the other *Fusarium* isolates were slow growing. The mycelium of *F. decemcellulare* was initially white then turning cream to pinkish-red due to a pinkish or reddish pigmentation produced in the agar (Figure 25). The cultures later produced yellow sporodochia from which droplets of exudates formed to give the colony a moist appearance. Microconidia were abundant in the aerial mycelia of younger colonies, and were oval to cylindrical, hyaline, typically thin walled and 0-1 septate. Macroconidia were hyaline, sickle shaped, generally 5 to 9 septate and 65.8 to 66.0 × 9.1 to 9.2 µm (Figure 26). The other *Fusarium* spp. produced fluffy creamy white to pink concentric-ringed colonies (Figure 27) and their macroconidia (3 to 6 septate) (Figure 28) measured 34.8 to 62.0 × 3.0 to 5.0 µm (Table 4).

Figure 25. Colony appearance of 14-day old cultures of *F. decemcellulare* IMI380504. Photo: R. A-A

Figure 26. Macroconidia of *F. decemcellulare* IMI380504. (Bar = 10µm) Photo: R. A-A

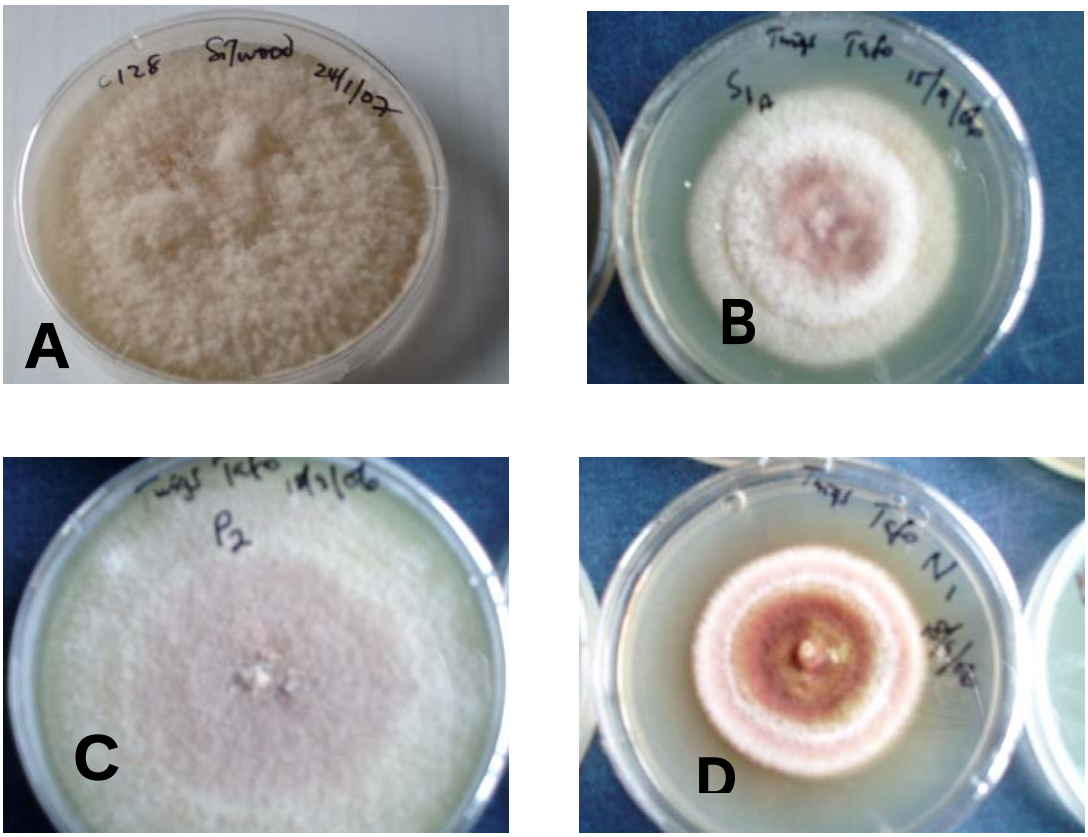

Figure 27. Colony appearance of 14-day old cultures from some *Fusarium* isolate cultures: A, AC748; B, AC511; C, AC110; D, AC995.

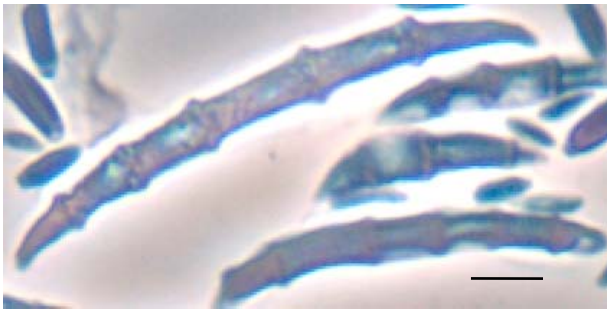

Figure 28 Macroconidia of *Fusarium* sp. Isolate AC995 (bar = 10µm)
Photo: R. A-A

The *Lasiodiplodia* isolates on the other hand, presented little variation in mycelium colour. The colonies were fast growing (section 2.4.2) and, in a comparative study of features of growth and sporulation under light and dark conditions, *L. theobromae* colonies were dense with an appressed mycelial mat. The aerial mycelium was initially white, turning greyish with age under light conditions, or sooty black when grown in continuous darkness (Figure 29). Small black raised fruiting bodies

(pycnidia) of the fungus were produced in the presence of light and would develop as superficial outgrowths. They were mostly solitary, conical, cylindrical or roundish and shiny black. The young conidia of *L. theobromae* were hyaline and unicellular, and in shape, rounded to ellipsoidal. Mature conidia were two-celled, dark brown in colour (Figure 30), and measured 21.9 to 22.4 × 11.9 to 12.2 µm. The cultural characteristics of all 36 *L. theobromae* obtained from infected cocoa tissue from Ghana were similar on PDA and identical to reference isolate IMI333797 identified by CABI and spore dimensions were consistent between replicates and in daughter colonies. The observed characteristics conform to published descriptions of the species (Pavlic *et al.*, 2004).

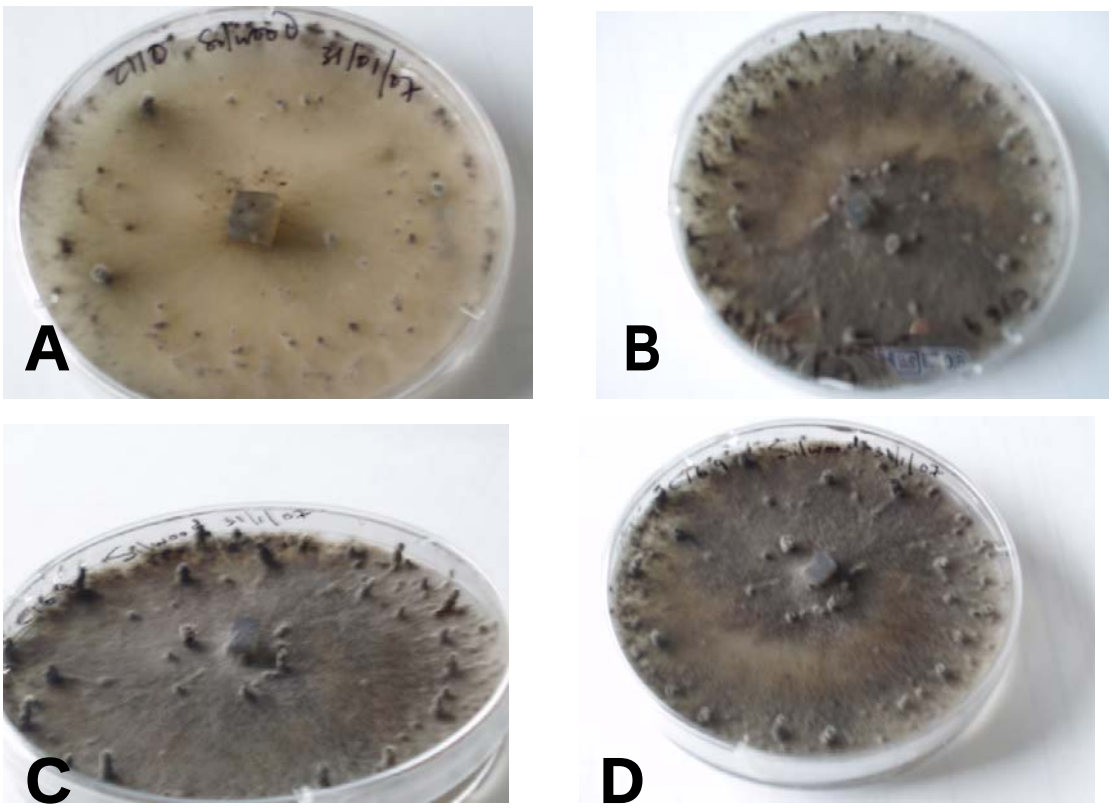

Figure 29. Colony appearance of some *Lasiodiplodia* isolates grown under 12hour alternating light and darkness. A, AC 008. B, AC 371. C, IMI 333797. D, AC 972. Photo: R. A-A.

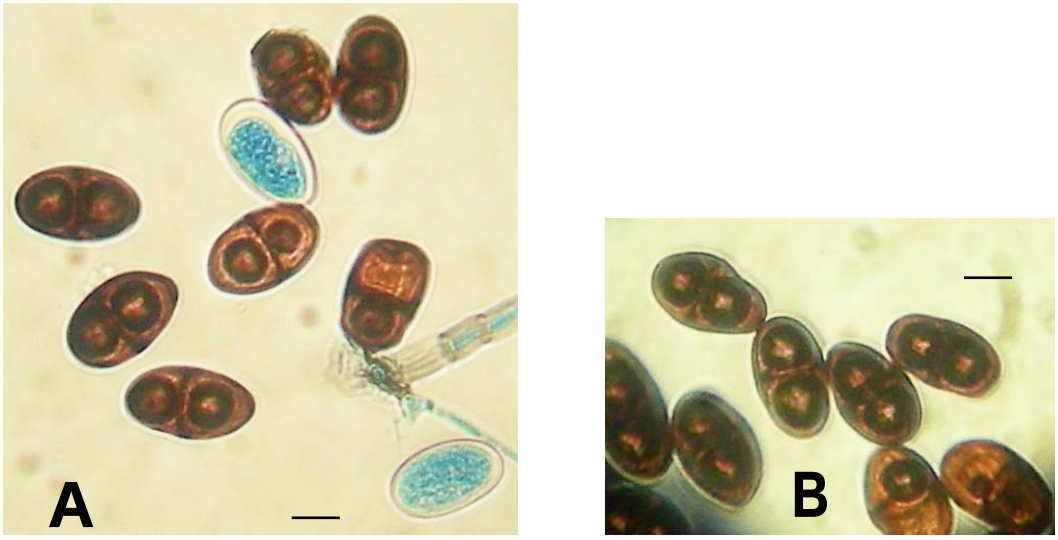

Figure 30. Conidia of some *L. theobromae* isolates grown under light conditions. A, AC 371. B, IMI 333797. Photo: R. A-A. (Bar =10 µm)

Table 5. Suitability of different media for isolation and support of growth of different fungi from infected cocoa stems (n = 82).

Fungal isolates	Growth on			
	PDA	PCA	CLA	CPHA
Fusarium spp.	++++	+++	+	++
L. theobromae	++++	++++	+	+++

+ Poor colony; ++ moderate colony; +++ good; ++++ very good colony.

2.4.2. Growth rate studies

All isolates grew within the range 15 to 35 °C In general, radial growth rates in *L. theobromae* isolates were higher (14.75 mm per day) than in *F. decemcellulare* (1.50 mm per day) or the other *Fusarium* spp. (2.00 mm per day). Overall, the different isolates studied had growth optima at 30 °C (Figure 31). Results from a mean of five replicates are presented in Figure 31 which are representative of the overall results of all the isolates on PDA at the temperatures studied (see Appendix 1).

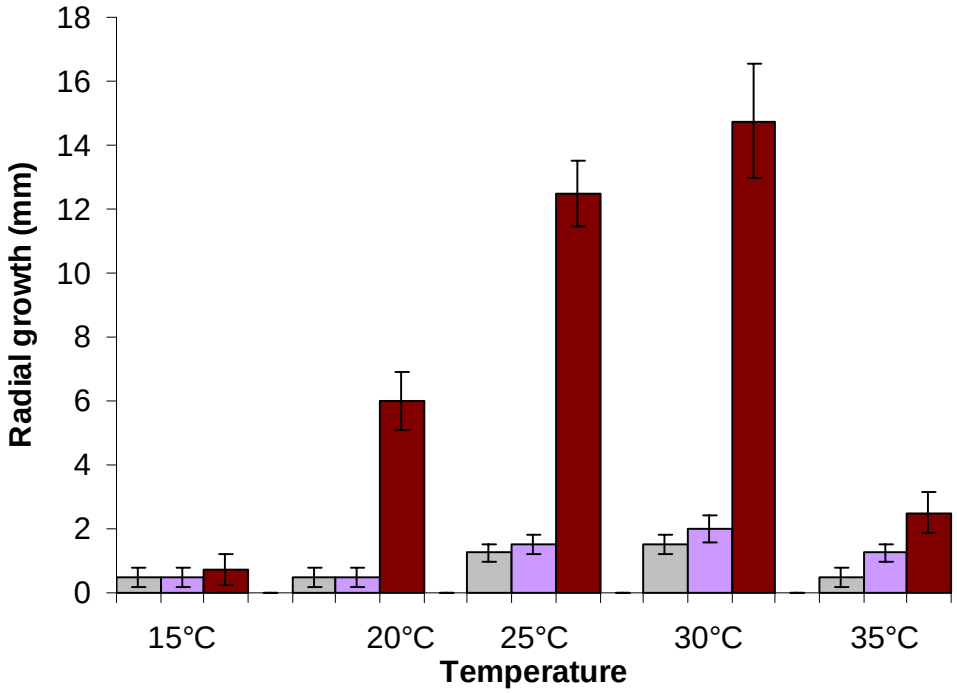

Figure 31. Radial growth rates (mm per 24 hours) of fungi from cocoa stems. Mean of 5 replicates. Bar indicates standard error.

2.4.3. The longevity of conidia in culture

The results of spore germination tests on five isolates including isolates of *L. theobromae*, *F. decemcellulare* and the other *Fusarium* spp. at different aging times are given in Figure 32. Significant differences in conidial viability over time were detected for each of the isolates utilised in this study and there was a steady decline in spore germination. Loss of viability was approximately linear with time, (Figure 33) with 60 % viability at 3 months declining to <10 % at 6 months. All the fungi lost viability of conidia at similar rates.

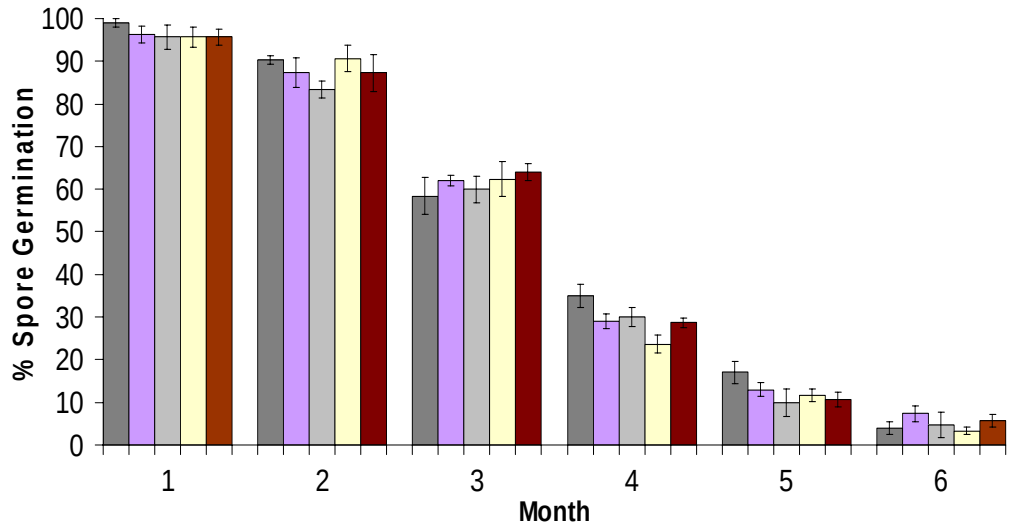

Figure 32. Graph showing percentage germination of conidia on PDA over time (Bar indicates standard error).

IMI380504 (F. decemcellulare) GJS0381 (F. decemcellulare) AC120 (F. chlamydosporum)
AC371 (L. pseudotheobromae) IMI333797 (L. pseudotheobromae)

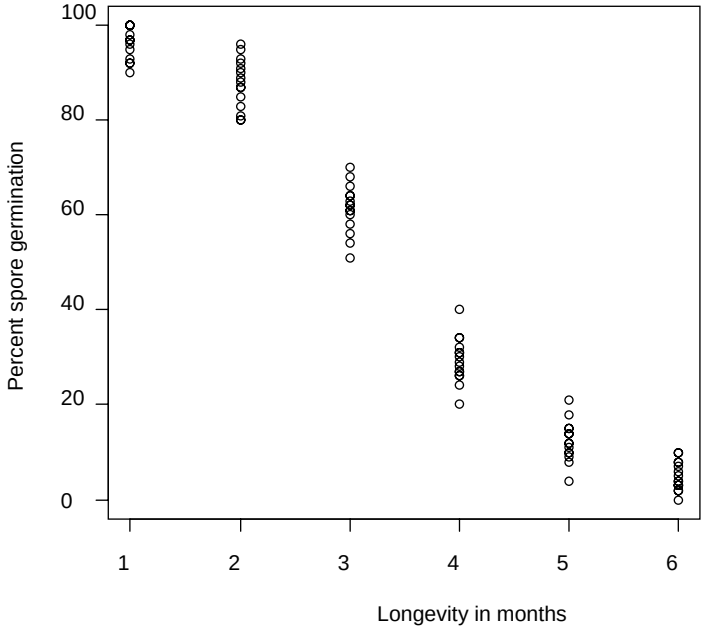

Figure 33. Pearson's product-moment correlation of percentage spore germination of five isolates against longevity in months r = -0.9, t = -41.9688, df = 88, p-value = <2.2e-16; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from -0.9841157 to -0.9635682.

2.4.4. Competition between isolates in culture

There was evidence of differential competition between *L. theobromae* and the two species of *Fusarium* when they were grown *in-vitro* on PDA plates. When singly grown, *L. theobromae* colonies grew faster than either single-grown *F. decemcellulare* or *Fusarium* spp. colonies. In dual culture, colonies of *F. decemcellulare* and *Fusarium* spp. grew at similar rates and each covered 50 % area of the PDA plate. Nevertheless, growth of both *F. decemcellulare* and *Fusarium* spp. colonies was halted when they approached a colony of *L. theobromae* (Figure 34). The margin of *L. theobromae* colonies then became thicker, with aerial hyphae forming a ridge-like barrier between the two following which *L. theobromae* eventually completely overgrew colonies of *F. decemcellulare* and *Fusarium* spp.

Figure 34. Competition between isolates in culture (the creamy-pink spot on the left plate is *F. decemcellulare* IMI380504 while that on the right plate is *Fusarium* spp. AC705. The dark spots to the right on both plates represent isolate *Lasiodiplodia theobromae* AC371).

2.4.5. Pathogenicity tests

2.4.5.1. Mycelium plug inoculation test

A total of seventy-eight isolates induced dieback symptoms on Amelonado cocoa in this preliminary test. The number included all 36 *L. theobromae* from cocoa, the reference *L. theobromae* isolate from CABI (IMI333797), four *F. decemcellulare* reference isolates, two each from CABI (IMI 380504 & IMI 361352) and USDA (GJS03-81 & GJS01-170). The aggregate of *Fusarium* spp. contained relatively fewer pathogenic isolates (37 representing 45.6 %). Thus, forty-four of the *Fusarium* isolates produced neither visible nor systemic symptoms, as the inoculated plants grew at the same rate as the controls. Moreover, recovery experiments yielded no growth in culture, indicating that these fungi had not established in the plants. The number of days for symptom expression and dieback scores are shown in Table 6. Symptoms of disease were similar for the different fungi including wilting and drooping of leaves, browning of vascular tissue (Figure 35), and death of the apical shoot back into older tissue. Ultimately, the leaves dried and in some plants detached from the branches leaving a leafless stem. Lesions on main stems ranged from dark brown to black colouration at wounding sites of all inoculated plants and they spread both up and down the stem from the inoculation site, and the more pathogenic isolates could kill the plant in four weeks. On primary branches, however, lesions spread both up and down from inoculation sites but dieback occurred only on the inoculated branch. Lesions on these stems (branches) were dark brown to black in colour. Mean number of days for disease symptoms to appear (incubation period) and the severity of dieback were recorded for each isolate after 12 weeks (Table 6). Isolates with the greatest lesion size were considered most pathogenic whilst isolates with the smallest lesion size were considered least pathogenic. Isolates that produced neither lesions nor dieback were considered non pathogenic. An analysis of variance showed that there were significant differences among the isolates ($P \leq 0.05$).

The control plants and those plants inoculated with one *Cephalosporium* isolate and some 44 *Fusarium* isolates grew normally during the assay period. These healthy plants continued to produce new flush leaves and stem growth was visible. They

showed no symptoms of chlorosis, wilting nor canker formation and all re-isolations were negative. One other CABI isolate that was supplied as a *Lasiodiplodia* from mango (IMI297320) was non pathogenic and did not resemble the fungus in any respect. Overall re-isolation success was 80 % and included at least one successful recovery for each isolate. Failure to recover the pathogens was commonly associated with the presence of 'contaminant' microorganisms, principally *Penicillium* spp. that were also isolated from many wounds inoculated with sterile agar. Culturing of the re-isolated fungi produced the same type of cultures as those from which the inoculations were made, and when they were re-inoculated onto healthy plants, they produced similar symptoms. It was assumed, therefore, that the disease observed on the inoculated plants was caused by the fungal isolates, satisfying Koch's postulates.

Figure 35. Internal tissues of an inoculated plant (left) and a control plant (right).

Table 6. Mean incubation period (inoculation to symptom appearance in days) and dieback incidence (present or absent) on 2 month-old Amelonado seedlings inoculated (agar plug) with a range of isolates of *Fusarium* and *Lasiodiplodia.* Data were taken 12 weeks after inoculation.

Isolates	Host of isolation	Identity	Days to symptom expression	Dieback disease development[1]
IMI 380504	*Ceiba pentandra*	*F. decemcellulare*	16-20	++
IMI 361352	*T. cacao*	*F. decemcellulare*	10-18	+++
IMI 333797	*Manihot esculenta*	*L. theobromae*	7- 15	+++
IMI 297320	*Mangifera indica*	*L. theobromae?*		-
GJS03-81	*T. cacao*	*F. decemcellulare*	7-17	+++
GJS01-170	*T. cacao*	*F. decemcellulare*	12-23	++
AC581	*T. cacao*	*L. theobromae*	6-10	+++
AC375	*T. cacao*	*L. theobromae*	5-10	+++
AC639	*T. cacao*	*L. theobromae*	6-12	+++
AC680	*T. cacao*	*L. theobromae*	6-10	+++
AC640	*T. cacao*	*L. theobromae*	6-10	+++
AC742	*T. cacao*	*L. theobromae*	7-10	+++
AC008	*T. cacao*	*L. theobromae*	8-13	+++
AC857	*T. cacao*	*L. theobromae*	8-12	+++
AC718	*T. cacao*	*L. theobromae*	9-13	+++
AC523	*T. cacao*	*L. theobromae*	8-11	+++
AC036	*T. cacao*	*L. theobromae*	7-13	+++
AC845	*T. cacao*	*L. theobromae*	11-13	+++
AC456	*T. cacao*	*L. theobromae*	11-16	+++
AC496	*T. cacao*	*L. theobromae*	10-14	+++
AC420	*T. cacao*	*L. theobromae*	11-16	+++
AC673	*T. cacao*	*L. theobromae*	11-17	+++
AC284	*T. cacao*	*Fusarium*	7-14	+
AC593	*T. cacao*	*Fusarium*	6-13	+
AC255	*T. cacao*	*Fusarium*	8-14	+
AC197	*T. cacao*	*Fusarium*	11-17	++
AC914	*T. cacao*	*Fusarium*	7-12	+
AC110	*T. cacao*	*Fusarium*	7-11	+
AC174	*T. cacao*	*Fusarium*	8-18	+
AC705	*T. cacao*	*Fusarium*	9-13	+
AC328	*T. cacao*	*Fusarium*	8-10	+
AC402	*T. cacao*	*Fusarium*	7-14	+
AC773	*T. cacao*	*Fusarium*	7-16	+
AC768	*T. cacao*	*Fusarium*	8-20	+++
AC219	*T. cacao*	*Fusarium*	9-17	+
AC739	*T. cacao*	*Fusarium*	8-11	+
AC580	*T. cacao*	*Fusarium*	8-17	+++
AC211	*T. cacao*	*Fusarium*	9-15	++
AC076	*T. cacao*	*Fusarium*	10-16	+++
AC551	*T. cacao*	*Fusarium*	9-25	+++
AC361	*T. cacao*	*Fusarium*	9-19	+++
AC451	*T. cacao*	*Fusarium*	10-19	++
AC330	*T. cacao*	*Fusarium*	9-15	+
AC037	*T. cacao*	*Fusarium*	9-18	+
AC120	*T. cacao*	*Fusarium*	9-14	+
AC229	*T. cacao*	*Fusarium*	8-15	++
AC511	*T. cacao*	*Fusarium*	8-12	+
AC317	*T. cacao*	*Fusarium*	9-11	+
AC206	*T. cacao*	*Fusarium*	8-12	+
AC787	*T. cacao*	*Fusarium*	8-15	+
AC806	*T. cacao*	*Fusarium*	10-14	+
AC845	*T. cacao*	*L. theobromae*	12-18	++
AC810	*T. cacao*	*L. theobromae*	11-15	+++
AC220	*T. cacao*	*L. theobromae*	13-20	+
AC644	*T. cacao*	*L. theobromae*	13-16	+
AC748	*T. cacao*	*Fusarium*	14-21	+
AC767	*T. cacao*	*Fusarium*	10-17	+
AC392	*T. cacao*	*L. theobromae*	11-14	+++
AC031	*T. cacao*	*Fusarium*	11-14	+
AC068	*T. cacao*	*L. theobromae*	12-16	+++
AC564	*T. cacao*	*L. theobromae*	11-16	+
AC972	*T. cacao*	*L. theobromae*	10-17	+
AC360	*T. cacao*	*L. theobromae*	12-15	+
AC280	*T. cacao*	*L. theobromae*	10-14	+
AC450	*T. cacao*	*L. theobromae*	11-17	+
AC371	*T. cacao*	*L. theobromae*	10-17	+
AC536	*T. cacao*	*L. theobromae*	12-16	+

AC329	*T. cacao*	*L. theobromae*	5-10	+
AC568	*T. cacao*	*L. theobromae*	7-11	+
AC064	*T. cacao*	*L. theobromae*	4-12	++
AC492	*T. cacao*	*L. theobromae*	7-14	+++
AC318	*T. cacao*	*L. theobromae*	13-21	+++
AC656	*T. cacao*	*Fusarium*	8-14	+
AC276	*T. cacao*	*Fusarium*	7-13	+
AC322	*T. cacao*	*L. theobromae*	12-18	+
AC407	*T. cacao*	*L. theobromae*	13-21	+++
AC383	*T. cacao*	*L. theobromae*	8-19	+++
AC296	*T. cacao*	*Fusarium*		-
AC714	*T. cacao*	*Fusarium*		-
AC274	*T. cacao*	*Fusarium*		-
AC114	*T. cacao*	*Fusarium*		-
AC226	*T. cacao*	*Fusarium*		-
AC113	*T. cacao*	*Fusarium*		-
AC965	*T. cacao*	*Fusarium*		-
AC081	*T. cacao*	*Fusarium*		-
AC670	*T. cacao*	*Fusarium*		-
AC695	*T. cacao*	*Fusarium*		-
AC209	*T. cacao*	*Fusarium*		-
AC844	*T. cacao*	*Fusarium*		-
AC243	*T. cacao*	*Fusarium*		-
AC583	*T. cacao*	*Fusarium*		-
AC346	*T. cacao*	*Fusarium*		-
AC521	*T. cacao*	*Fusarium*		-
AC770	*T. cacao*	*Fusarium*		-
AC489	*T. cacao*	*Fusarium*		-
AC820	*T. cacao*	*Fusarium*		-
AC231	*T. cacao*	*Fusarium*		-
AC514	*T. cacao*	*Fusarium*		-
AC972	*T. cacao*	*Fusarium*		-
AC964	*T. cacao*	*Fusarium*		-
AC956	*T. cacao*	*Fusarium*		-
AC680	*T. cacao*	*Fusarium*		-
AC638	*T. cacao*	*Fusarium*		-
AC275	*T. cacao*	*Fusarium*		-
AC335	*T. cacao*	*Fusarium*		-
AC645	*T. cacao*	*Fusarium*		-
AC539	*T. cacao*	*Fusarium*		-
AC108	*T. cacao*	*Fusarium*		-
AC424	*T. cacao*	*Cephalosporium?*		-
AC619	*T. cacao*	*Fusarium*		-
AC881	*T. cacao*	*Fusarium*		-
AC418	*T. cacao*	*Fusarium*		-
AC274	*T. cacao*	*Fusarium*		-
AC856	*T. cacao*	*Fusarium*		-
AC803	*T. cacao*	*Fusarium*		-
AC377	*T. cacao*	*Fusarium*		-
AC668	*T. cacao*	*Fusarium*		-
AC381	*T. cacao*	*Fusarium*		-
AC291	*T. cacao*	*Fusarium*		-
AC367	*T. cacao*	*Fusarium*		-
AC614	*T. cacao*	*Fusarium*		-
AC774	*T. cacao*	*Fusarium*		-

[1]- Nil, + poor, ++ fair, and +++ severe.

2.4.5.2. Spore inoculation test

Six hours after inoculation onto Amelonado seedlings using conidia, nearly all spores had germinated and germ tubes had formed as indicated by microscopic observation. By 24 hours, hyphal growth had increased and attempted penetrations were visible. Plants were observed for signs of disease for periods up to 12 weeks. Adverse effects of wounding were minimal or absent in all plants. Consistent with the mycelium plug inoculation test, all isolates previously scored as pathogenic caused disease when inoculated with spores.

At the point of inoculation, a wound tissue reaction of gumming and swelling occurred, and both the inoculated and control plants showed the same pattern of formation of callus tissue and complete healing. By the 35th day after inoculation, the pathogenic isolates had largely established, though the internal growth of *Fusarium* spp. was somewhat lower than *Lasiodiplodia* since recovery isolation by subculturing always yielded a lower proportion of positives than with *F. decemcellulare* and *L. theobromae.* The agreement in effectiveness among 37 isolates of *Fusarium* in inducing wood necrosis and dieback disease in Amelonado seedlings indicates that these species of *Fusarium* are truly pathogenic. All 37 *L. theobromae* isolates including the reference isolate were able to cause dieback symptoms on Amelonado cocoa seedlings. The susceptibility of Amelonado seedlings to the 78 different fungal isolates using spore inoculation is summarised in Table 7. The control plants developed no visible disease symptoms and the inoculation sites of the seedlings were closed by callus tissue after 12 weeks. From parallel trials (section 2.4.5.1) it was evident that the seedlings inoculated with isolates from each group of pathogen (*F. decemcellulare*, *Fusarium* spp. and *L. theobromae*) became equally diseased irrespective of whether they were inoculated with mycelium plugs or spore suspension. In both cases plants drooped and eventually died. Spore concentration had a significant effect ($P \leq 0.05$) on the level of infection (Figure 36). At the lower end of the spore concentration series (1×10^3 per ml), there was no disease visible and plant growth was similar to that of the water-treated controls. However, a concentration of 1×10^6 conidia per ml reduced growth of the plants, and on the whole, 90 % of such

plants had a disease score of six or more. Mean dieback score in individual isolate treatments ranged from 5.2 to 9.0 for *Fusarium* and 7.7 to 9.0 for *L. theobromae.*

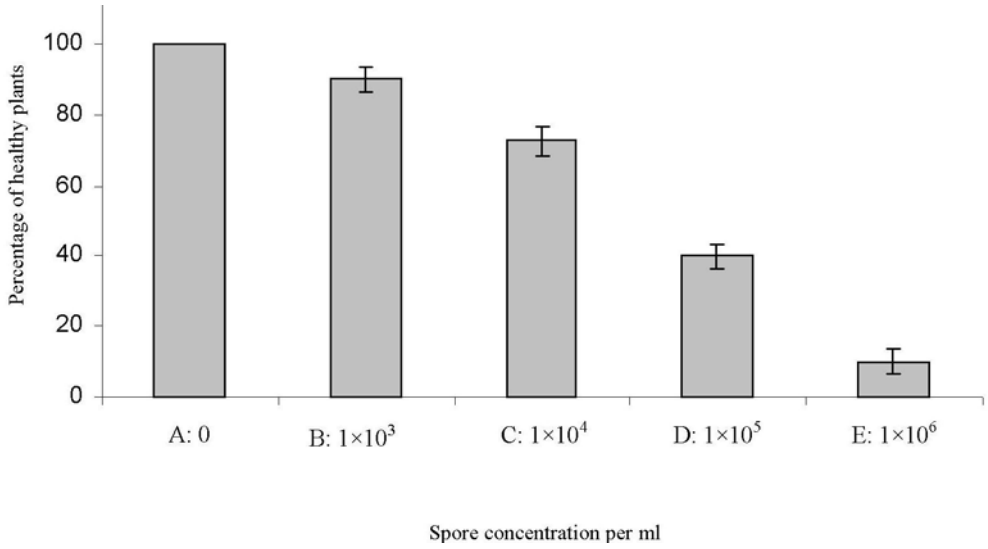

Figure 36. Effect of spore concentration of isolate IMI 380504 on disease development. The bars represent percentage of healthy plants 8 wks after inoculation. Bar indicates standard error.

Dieback symptoms appeared 9 to 25 days after inoculation. Thus, Amelonado showed a very susceptible reaction to 78 isolates tested using spore inoculation. *Fusarium decemcellulare*, *Fusarium* spp. and *L. theobromae* caused similar disease symptoms but there were significant differences ($P \leq 0.05$) between isolates in lesion sizes and dieback scores. The largest values were recorded for *L. theobromae* and the smallest for *Fusarium* spp. Mean lesion lengths caused by *L. theobromae* ranged from 37 mm to 51 mm, whereas those of *F. decemcellulare* ranged from 31 mm to 43 mm (Table 7). The initial signs of attack included yellowing and wilting of leaves followed by death of the tops. Occasionally, small necrotic lesions on the bark coalesced to form large necrotic patches on the stem. These are characteristic symptoms of the disease as it occurs in the field. Seventy-eight isolates (including those from CABI and USDA and all the *L. theobromae* isolates) induced dieback symptoms on Amelonado cocoa and were consistently re-isolated from the lesions on inoculated seedlings and never from control plants. To satisfy Koch's postulates, the re-isolated fungi from the inoculated plants were cultured on sterile PDA. They were found to produce the same type of cultures as those from which they came. When these strains were inoculated into healthy plants, they produced similar symptoms as before. This observation gives the strong impression that the disease commonly observed on diseased cocoa trees in

Ghana and those found on the inoculated plants was caused by these pathogens. It is worthy to mention that the proportion of 'contaminant' microorganisms, principally *Penicillium* spp. was high from some wounds.

Table 7. Pathogenicity following conidial inoculation of *Fusarium decemcellulare*, *Fusarium* spp. and *Lasiodiplodia theobromae* on West African Amelonado cocoa seedlings.

Fungus	Pathogenicity			
	Isolate	Days to visible infection	Mean lesion length (mm) ± s.e. [x]	Mean dieback score (1-9)¶
F. decemcellulare	IMI 380504	12-20	42.3 ± 2.35	9.0
	IMI 361352	14-23	41.2 ± 1.85	8.2
	GJS 03-81	18-24	31.8 ± 5.94	6.0
	GJS 01-170	14-18	43.5 ± 2.08	4.2
Fusarium species	AC 037	16-19	40.2 ± 2.92	4.5
	AC 110	14-23	38.0 ± 3.57	5.5
	AC 120	14-20	31.8 ± 5.76	3.0
	AC 174	12-16	26.2 ± 4.95	6.0
	AC 197	14-19	31.3 ± 2.84	6.0
	AC 206	13-23	31.6 ± 6.33	6.7
	AC 229	16-22	34.5 ± 5.96	6.0
	AC 255	13-17	29.5 ± 5.26	5.2
	AC 276	13-22	38.8 ± 2.93	6.7
	AC 284	14-22	29.1 ± 5.31	6.0
	AC 328	13-20	35.0 ± 2.26	3.0
	AC 330	13-19	37.4 ± 6.49	6.7
	AC 402	12-18	33.5 ± 1.51	6.0
	AC 451	12-19	34.6 ± 5.73	6.0
	AC 593	14-16	35.3 ± 6.57	6.0
	AC 638	13-15	34.3 ± 3.61	6.7
	AC 656	14-20	34.4 ± 6.36	6.0
	AC 705	14-24	37.9 ± 3.41	6.7
	AC 773	16-19	33.5 ± 2.36	6.0
	AC 914	12-19	39.7 ± 3.35	6.7
	AC 317	15-19	40.1 ± 2.20	7.5
	AC 511	13-17	33.4 ± 5.07	6.7
	AC 787	14-22	30.8 ± 5.10	6.0
	AC 806	13-22	30.6 ± 4.16	6.0
	AC 031	11-18	33.1 ± 1.55	3.0
	AC 392	17-22	30.2 ± 6.48	5.2
	AC 748	13-19	34.4 ± 4.73	3.0
	AC 767	14-18	44.9 ± 3.27	4.4
	AC 076	13-16	33.2 ± 3.16	6.0
	AC 211	19-26	30.0 ± 6.99	8.4
	AC 219	15-22	33.4 ± 3.83	6.7
	AC 361	13-25	36.0 ± 3.70	7.5
	AC 551	12-21	30.6 ± 6.71	6.0
	AC 580	13-20	32.3 ± 5.58	8.2
	AC 739	10-19	25.4 ± 3.89	7.5
	AC 768	12-17	33.6 ± 2.20	7.5
	AC 995	15-24	27.0 ± 6.65	6.2
Lasiodiplodia species	IMI 333797	12-20	45.9 ± 2.73	8.2
	AC 008	13-18	49.9 ± 1.80	9.0
	AC 036	12-18	41.6 ± 3.51	7.5
	AC 064	9-17	48.6 ± 0.46	9.0
	AC 068	12-16	44.1 ± 3.23	8.2
	AC 280	13-20	39.7 ± 4.89	7.5
	AC 318	10-14	45.4 ± 3.75	8.2
	AC 322	13-21	39.8 ± 3.88	7.5
	AC 329	12-18	45.6 ± 3.85	8.2
	AC 360	8-16	48.0 ± 0.75	9.0
	AC 371	12-15	43.6 ± 3.52	8.2
	AC 375	10-17	51.9 ± 1.32	9.0
	AC 383	10-15	39.7 ± 4.69	7.5
	AC 407	13-18	49.7 ± 1.62	9.0
	AC 450	13-21	44.1 ± 3.05	8.2
	AC 492	11-16	44.4 ± 3.23	8.2
	AC 536	11-17	46.7 ± 2.12	9.0
	AC 564	11-16	44.2 ± 3.87	8.2
	AC 568	12-18	38.4 ± 4.34	6.7
	AC 581	9-14	44.2 ± 1.54	8.2
	AC 640	10-17	37.8 ± 5.69	7.5
	AC 644	12-16	35.2 ± 3.03	7.5
	AC 680	12-16	44.4 ± 2.68	8.2
	AC 742	11-16	42.1 ± 3.25	7.5
	AC 810	10-15	37.3 ± 5.31	7.5
	AC 845	11-15	45.8 ± 2.86	8.2
	AC 857	11-17	39.1 ± 4.20	8.2
	AC 972	11-15	43.7 ± 1.34	9.0
	AC 220	13-16	46.1 ± 1.52	9.0
	AC 420	11-17	42.2 ± 5.47	8.2
	AC 456	11-15	38.1± 5.27	8.2
	AC 487	14-18	34.0 ± 5.50	7.5
	AC 496	11-16	41.1 ± 1.14	6.7
	AC523	11-15	39.6 ± 1.57	8.2
	AC 639	11-13	49.1 ± 2.67	9.0
	AC 673	12-17	38.3 ± 2.12	8.2
	AC 718	10-17	47.2 ± 3.29	8.2

¶ Mean dieback score = dieback disease score recorded after 6 weeks on a 1-9 scale, where 1 = 0 % infection with no visible infection and 9 = 100% infection or complete plant death.[x]

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2.4.5.3. Effect of humidity on disease development

There was a significant effect of post-inoculation high relative humidity on dieback infection caused by isolates AC371, AC845, IMI380505 and IMI361352. For all isolates there was a strong trend of high disease incidence with high humidity possibly due to increased spore germination (Figure 37) and germ tube penetration after 12 h incubation in the dew chamber. In the low humidity treatment, the spore suspensions were dry after 12 hours and spore germination and subsequent penetration were dramatically reduced.

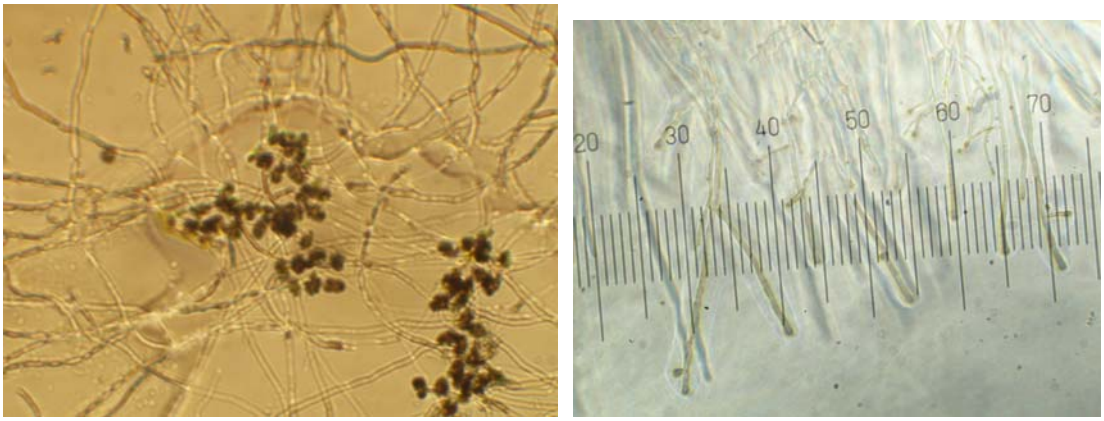

Figure 37. Typical conidia germination *in-vitro* 12 hours after inoculation: left = *L. theobromae* (AC371) and right photo = *F. decemcellulare* (IMI380504).

In the higher relative humidity environment the plants produced early disease symptoms and a greater proportion of inoculated plants became infected while more time was required to produce the same symptoms in plants kept at low humidity. The latter showed early symptoms only after 18 days (Table 8). Thirty-nine of the 40 plants held at high humidity compared with three in the low humidity environment developed signs of dieback. Thus, the results showed that growth and development of the pathogens within the stem, and the induction of dieback were significantly enhanced by high air humidity but the effect of soil water status seemed to be minimal.

Table 8. Effect of varying relative humidity and soil moisture on disease development.

Relative humidity range[1]	Number of seedlings tested per isolate	Fungal isolates							
		AC371		AC 845		IMI361352		IMI380504	
		First symptoms (days)	No. of diseased plants	First symptoms (days)	No. of diseased plants	First symptoms (days)	No. of diseased plants	First symptoms (days)	No. of diseased plants
HrHs	5	6	5	7	5	10	4	7	5
HrLs	5	9	5	8	5	10	5	9	5
LrHs	5	22	1	18	1	-	0	-	0
LrLs	5	25	1	-	0	-	0	-	0

[1]HrHs = high relative humidity, high soil moisture; HrLs = high relative humidity, low soil moisture; LrHs = low relative humidity, high soil moisture; LrLs = low relative humidity, low soil moisture.

2.4.5.4. Inoculum type, access and inoculation of different plant parts on disease development

To determine the most effective route(s) of entry of the pathogens into cocoa, different plant parts were variously inoculated as described above and observed for disease symptoms. Petioles, pulvini and intact leaves were not infected with any of the tested isolates despite being in contact with spores or agar plugs for more than 28 days whereas placing the inoculum on needle or scalpel wounds on the stem resulted in 100 per cent infection (Table 9). Inoculation sites that were not wounded supported some superficial hyphal growth but never became infected. It was apparent that the means by which conidia are brought in contact with the xylem strongly influences the development of disease. It is suggested that the comparatively large amount of damage caused by scalpel incision compared with needle wounding may have added to the success of the mycelium plug method. Nevertheless, frequencies of dieback formation were similar for both pathogenic species (*F. decemcellulare* and *L. theobromae*).

On the excised tissue discs, spores of both *L. theobromae* and *F. decemcellulare* germinated and infected the stems but not the leaf discs. Thus fungal hyphae were observed neither in the leaf cells nor in the veins (Figure 38). The cells in inoculated tissues did not differ visibly from those in uninfected cells. In inoculated leaf discs of Amelonado, black spots occurred in some cases. Since these black dots were absent

from tissues of non-infected plants it is assumed that they represent plant responses such as hypersensitive reaction.

Table 9. Effects of inoculation method, inoculum type and plant part in Amelonado seedlings on infection success.

Isolate	Plant part	Number of seedlings tested	Inoculation method	Number of seedlings with dieback
AC371	Stem	3	Wound, mycelium plug	3
	Stem	3	Wound, spores	3
	Stem	3	No wound, mycelium plug	0
	Stem	3	No wound spores	0
	Intact leaf	3	Wound, mycelium plug	0
	Intact leaf	3	Wound, spores	0
	Intact leaf	3	No wound, mycelium plug	0
	Intact leaf	3	No wound spores	0
	Petiole	3	Wound, mycelium plug	0
	Petiole	3	Wound, spores	0
	Petiole	3	No wound, mycelium plug	0
	Petiole	3	No wound spores	0
	Pulvini	3	Wound, mycelium plug	0
	Pulvini	3	Wound, spores	0
	Pulvini	3	No wound, mycelium plug	0
	Pulvini	3	No wound spores	0
AC845	Stem	3	Wound, mycelium plug	2
	Stem	3	Wound, spores	3
	Stem	3	No wound, mycelium plug	0
	Stem	3	No wound spores	0
	Intact leaf	3	Wound, mycelium plug	0
	Intact leaf	3	Wound, spores	0
	Intact leaf	3	No wound, mycelium plug	0
	Intact leaf	3	No wound spores	0
	Petiole	3	Wound, mycelium plug	0
	Petiole	3	Wound, spores	0
	Petiole	3	No wound, mycelium plug	0
	Petiole	3	No wound spores	0
	Pulvini	3	Wound, mycelium plug	0
	Pulvini	3	Wound, spores	0
	Pulvini	3	No wound, mycelium plug	0
	Pulvini	3	No wound spores	0
IMI 380504	Stem	3	Wound, mycelium plug	3
	Stem	3	Wound, spores	3
	Stem	3	No wound, mycelium plug	0
	Stem	3	No wound spores	0
	Intact leaf	3	Wound, mycelium plug	0
	Intact leaf	3	Wound, spores	0
	Intact leaf	3	No wound, mycelium plug	0
	Intact leaf	3	No wound spores	0
	Petiole	3	Wound, mycelium plug	0
	Petiole	3	Wound, spores	0
	Petiole	3	No wound, mycelium plug	0
	Petiole	3	No wound spores	0
	Pulvini	3	Wound, mycelium plug	0
	Pulvini	3	Wound, spores	0
	Pulvini	3	No wound, mycelium plug	0
	Pulvini	3	No wound spores	0
IMI 361 352	Stem	3	Wound, mycelium plug	2
	Stem	3	Wound, spores	2
	Stem	3	No wound, mycelium plug	0
	Stem	3	No wound spores	0
	Intact leaf	3	Wound, mycelium plug	0
	Intact leaf	3	Wound, spores	0
	Intact leaf	3	No wound, mycelium plug	0
	Intact leaf	3	No wound spores	0
	Petiole	3	Wound, mycelium plug	0
	Petiole	3	Wound, spores	0
	Petiole	3	No wound, mycelium plug	0
	Petiole	3	No wound spores	0
	Pulvini	3	Wound, mycelium plug	0
	Pulvini	3	Wound, spores	0
	Pulvini	3	No wound, mycelium plug	0
	Pulvini	3	No wound spores	0

2.4.5.5. Response of leaf and stem discs to inoculation with isolates of *F. decemcellulare* and *L. theobromae*

Differences were observed between infection of stem and leaf discs following inoculation with isolates of *F. decemcellulare* and *L. theobromae.* Brown necrotic lesions were microscopically visible in stem discs but not in the leaf discs although attempted penetration was seen on the latter (Figure 38). The mean lesion size for individual isolates ranged from 2.21 to 2.58 mm for *F. decemcellulare* while in *L. theobromae*, the range was 2.72 to 2.95 mm (Table 10).

Table 10. Leaf and stem disc necrosis (lesion length, mm) in response to spore inoculation with isolates of *F. decemcellulare* and *L. theobromae* isolates.

Fungus species	Isolate	Mean[1] lesion length in stem disc	Mean lesion length in leaf disc
F. decemcellulare	IMI361352	2.21± 0.22a	0.0
	IMI380504	2.58 ± 0.10a	0.0
L. theobromae	AC371	2.95 ± 0.12b	0.0
	AC845	2.72 ± 0.10b	0.0

[1]Mean from 15 measurements. Means followed by different letters are significantly different at P < 0.05 (Tukey HSD test).

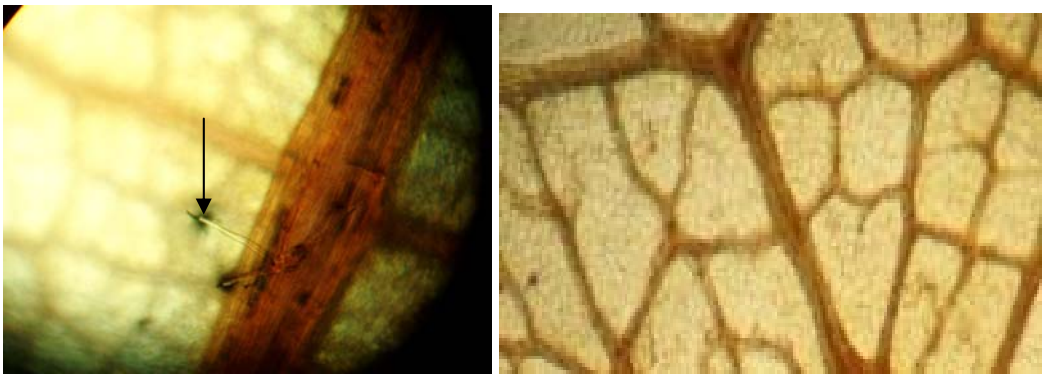

Figure 38. An inoculated site showing a penetrating germ tube (arrowed) and control water-inoculated leaf showing many intact cells. Photo: R. A-A.

2.5. Discussion

Cocoa dieback is reported as being caused by *F. decemcellulare* and *L. theobromae* (Crowdy, 1947; Owen, 1956). Currently, there is little information on the environmental requirements optimal for each pathogen, their relative frequency of occurrence and their relationships to each other *in-vivo*. Similarly, no information is available about the extent to which other fungi induce dieback disease in cocoa. The present results build on previous studies that presented a view of the occurrence and pathogenicity of *F. decemcellulare*: that this fungus is the main cause of dieback and that it remains active in wood for ten years or more, and can spread or cause disease if the tree becomes weakened in some way.

Different selective media have been used for fungal isolation, in particular for *Fusarium* (Tsao 1970; Thrane, 1996) with the objective of optimising isolation and enumeration. The most widely used medium has been of Nash and Snyder (1962) employing the soil fungicide PCNB, which partially inhibits many fungal contaminants but allows the normal development of *Fusarium* spp. Other selective agents used in *Fusarium* isolation media are dichloran (Andrews and Pitt, 1986) a mixture of dichloran and iprodione (Abildgren *et al.,* 1987), and Rose Bengal, benomyl, and captan (Elad and Chet, 1983), but all are known to allow the growth of some other fungal taxa. Only malachite green has been reported to be highly selective for *Fusarium* spp. and restrictive to other microorganisms (Singh and Nene, 1965). Among the media used in the current study, the fungi grew best on PDA medium followed by PCA with carnation leaf agar and cocoa pod husk agar being the poorest. The reason for this is unknown, but the high carbohydrate status of PDA with rich constituents may have played a part. PDA is a complex medium that includes dextrose and soluble starch and proteins. The use of complex selective media (other than the addition of streptomycin) was not judged necessary in the present investigation since, compared with soil, cocoa stem is already a selective substrate allowing the growth of relatively few species.

The fungi obtained from cocoa proved easy to handle. Thirty-six of them were *L. theobromae* while the remainder were *Fusarium* spp. None of these isolates was

identical to *F. decemcellulare*, but there is no doubt that the spores of *Fusarium* and *L. theobromae* are present at all periods during the development of dieback in cocoa plants. This was in contrast to observations published previously from Ghana by Crowdy (1947) and Owen (1956), citing *F. decemcellulare* as the most frequently isolated fungal pathogen from dieback lesions. The deadly effects of *F. decemcellulare* have received a good deal of attention in the past and the absence of the fungus in infected wood in the current study is surprising. The present role of *F. decemcellulare* in dieback disease in Ghana is not certain, as there is no obvious reason for its absence from the infected tissues collected from the field. Possibly, changes in fungal succession over the years might have affected occurrence but this must await a better understanding of how succession in fungi occurs in the cocoa ecosystem in relation to dieback disease. Only isolates from diseased trees from the Eastern and Western Regions of Ghana were available in the present study and I hope these findings will encourage others to develop comparative studies across a wider geographical range. Thus, future studies need to consider collection of isolates in other regions and should strive to sample all accessible cocoa growing areas. Such tests will increase our understanding of the present role that *F. decemcellulare* has in cocoa dieback disease in Ghana and other West African cocoa-growing regions. Though some differences in frequencies of isolation between *F. decemcellulare* and *L. theobromae* were reported earlier (Crowdy, 1947; Owen, 1956), these were not related to pathogenic ability. Owen (1956), for instance, made a number of fungal isolations from diseased cocoa in the field and found significant variation in the proportions in which *F. decemcellulare* and *L. theobromae* occurred. Further, at that time, the importance of other *Fusarium* species as being responsible for dieback was not recognised and emphasis was only on *F. decemcellulare.* It is possible that other *Fusarium* species were present in earlier studies but remained unnoticed due to the more aggressive pathogenicity (Table 7) of *F. decemcellulare.*

Substrate use patterns *in-vivo* may also be linked to changes in abundance or occurrence of the different fungi. In *in-vitro* studies, the interaction among the three fungal groups was shown to be competitive and the rather reduced growth of *Fusarium* could be related to nutritional stress due to competition with the more aggressive *Lasiodiplodia.* In the case of *F. decemcellulare* and *Fusarium* spp., both fungi competed for the substratum though not directly challenging to the other of the

two fungi. Their colonies overlapped to some extent. However, *L. theobromae* was aggressive and inhibited the growth of both *F. decemcellulare* and *Fusarium* spp. The aggressiveness of *L. theobromae* on the woody tissue of cocoa may significantly have limited the presence of *F. decemcellulare* from the fields surveyed, therefore establishing the dominance of the former pathogen. At present the interactions between the fungal groups *in-vivo* can only be surmised and may differ substantially from that seen on agar. The apparent decline of *F. decemcellulare* might be linked to the presence of these newly detected *Fusarium* species, including those apparently non-pathogenic on their own. A study of competitiveness between fungi in cocoa stem tissue might be a fertile area for investigating possible biocontrol in the future.

The dominant occurrence of *L. theobromae* over *F. decemcellulare* may also be partly explained by the practice in Ghana nowadays where farmers use cassava (*Manihot esculenta* Crantz) as a temporary shade crop during the establishment of new cocoa farms (Osei-Bonsu *et al.,* 1998). Besides being a revenue-generating crop, cassava may also have complementary effects on dieback disease of cocoa. For example, after cassava is harvested the residue is left to decompose in the soil and the decomposition of the tuberous roots can support prolific growth and sporulation of *L. theobromae* macroconidia (Onyeka *et al.*, 2005). If planted into a farm with a history of cassava, or if cassava is used for temporary shading, young cocoa will be exposed to an elevated number of infective macroconidia of *L. theobromae,* as cassava is known to be a host of this pathogen.

The morphology of *F. decemcellulare* isolates from Brazil and Cameroon (Table 4) was indistinguishable from that from Ghana obtained from CABI. The microconidia of all of them were oval and non-septate. Chlamydospores were absent and the fungus grew very slowly on artificial media. *Fusarium decemcellulare* produced a mixture of pink and red pigments in the agar, and most characteristically, the cultures produced yellow sporodochia from which droplets of exudate formed to give the colony a moist appearance. This observation conforms to the characteristics published by Leslie and Summerell (2006). Moreover, conidial measurements were within the size ranges published by Crowdy (1947). The other *Fusarium* isolates, however, differed from *F. decemcellulare* in that their colonies were white to creamy-pink with considerable differences in spore morphology. Their macroconidia were smaller than those of *F.*

decemcellulare but with a continuum of dimensions that gave no clues to identity or whether a single or multiple species were represented.

A study of the longevity of a selection of isolates from the different groups revealed that these fungi are short-lived on artificial media contrary to the observation by Crowdy (1947) that the macroconidia could survive in dead wood for up to 10 years. The maximum longevity of macroconidia on PDA at 25 ˚C was six months (Figure 32). Although the two environments are dissimilar the results suggest that re-investigation of longevity of inoculum in the field is needed.

In the pathogenicity tests, all three fungal groups acted as primary pathogens and that, to invade and to degrade the woody tissue of cocoa, they needed prior stem wounding. Spore germination (by microscopic observation) and host penetration occurred quickly after inoculation but isolates of *Fusarium* and *Lasiodiplodia* appeared to require up to 72 hours of continuous dew for maximum infection on cocoa at 27 ˚C. Temperature and humidity were probably the main environmental factors influencing the development of the fungi. In general the agreement between two inoculation methods was good, with the incubation period common to both inoculation methods ranging from 6 to 25 days (Table 6 and Table 7). Overall, either the needle and syringe application of spore suspensions or the use of wound and mycelium plugs gave comparable results when used for inoculation of Amelonado cocoa seedlings in the experimental work.

That *Fusarium* and *Lasiodiplodia* apparently required a wound to cause infection in cocoa can be associated with the common observation that insecticide applications aid in control of dieback in cocoa farms since mirid control probably reduces injuries on the stem and therefore reduces disease incidence of necrosis and dieback in cocoa. As it is not uncommon under field conditions for the incidence of dieback to be low even under very conducive conditions for disease development, it is most likely that mirid feeding lesions together with high spore levels are necessary to cause infections in the field. Sanitation by removal of infected chupons and/or branches before sporulation should be a very effective cultural practice to reduce dieback incidence in the field. The Amelonado cocoa variety evaluated in the present study is susceptible to both fungal groups and infection could spread from an inoculated main stem to non

inoculated branches but not the other way round. The reason for this observation is not clear at present and must be verified in future studies for information that would be useful for developing integrated management programmes for dieback disease of cocoa.

In the greenhouse, the average air temperature of 30.6 ± 4.3 ºC and a mean relative humidity of 65 % were conducive to infection and supported the development of dieback. The maintenance of high humidity in the greenhouse and the use of the dew chamber in the first three days after inoculation, helped to create the conditions needed for the survival of spores and infection of plants. The present findings also associate dieback disease incidence strongly to high air humidity in the surrounding environment. The light, temperature and relative humidity conditions experienced in the greenhouse were probably close to those tropical climatic conditions under which cocoa is grown. This presents a challenge for dieback management in West Africa where the weather is typically warm and humid in the rainy season (March to October), with average temperatures fluctuating around minimum, 22ºC (and maximum, 33 ˚C (Figure 1). Dieback incidence is often highest in July-October in Ghana, a time when the weather is usually cool and humid and this, coupled with intermittent wetness associated with rain, is likely to be conducive to fungal infection; the stem surface on which spores land may possibly have a film of water from rain or dew which can have a marked effect on the germination of spores.

Presence or absence of abundant soil moisture was not critical for disease development since the proportion of infected plants in dry soil was similar to those of drenched soil. The results support a hypothesis that drought conditions primarily inhibit spore germination, because a rise in dieback on cocoa farms is most noticeable during the rainy season. Evidence from other fungal diseases has repeatedly shown the importance of appropriate temperature and relative humidity to disease development. For example, a relative humidity above 87 % was essential for *Colletotrichum gloeosporioides* f. sp. *manihotis* Henn (Penn.) to cause anthracnose disease in cassava (Harrison and Williamson, 1994). In the case of the wheat pathogen *F. graminearum* macroconidia were produced at an optimal temperature of 28 to 32 ˚C and their production was inhibited below 16 ˚C and above 36 ˚C (Tschanz *et al*., 1976). On wheat spikelets, Andersen (1948) showed that millions of conidia of *F.*

graminearum were produced on moist wheat heads at 20 to 30 ˚C, and lesser numbers at 15 ˚C. Moreover, macroconidia appeared within 5 days at 20 ˚C and within 3 days at 25 to 30 ˚C. Exposure of spikelets to moisture reduced conidial formation time to one to 2 days, with conidial numbers increasing with increasing humidity. Examples of fungal infections where high humidity is essential for sporulation and/or infection are widely reported in the literature (Agrios, 2005).

In the current study the first characteristic symptoms of dieback were observed after a period that corresponded to the reported latent period of this disease under field conditions according to Crowdy (1947), thus indicating that the infection process in the greenhouse environment was similar to that expected in the field.

The testing of the isolates in the greenhouse against susceptible West African Amelonado cocoa seedlings was useful as a first step in the pathogenicity screening process; using this biological assay, those isolates with the ability to incite disease development were identified. The results selected a substantial number of potential pathogens, yet it succeeded in eliminating some 45 % of the *Fusarium* isolates as non-pathogenic. Significant differences were observed in pathogenicity among the isolates mainly between the *Fusarium* and *Lasiodiplodia.* The mean disease scores of isolates from *Fusarium* and *Lasiodiplodia* were 6.2 and 8.2, respectively, indicating that isolates of *Lasiodiplodia* were, on average, more aggressive than those of *Fusarium*. Significant variation ($P \leq 0.05$) within *F. decemcellulare* was also detected. For instance, isolate GJS 03-81, collected as a *Fusarium* endophyte from cocoa leaves in Brazil (Gary Samuels, personal communication) was less pathogenic than isolate GJS 01-170 collected as ascospores from Cameroon. However, no significant differences were detected ($P > 0.05$) between *Lasiodiplodia* collected from cocoa and the one isolate from cassava.

3. Chapter 3

3.1. Molecular characterisation of *Fusarium* and *Lasiodiplodia* species causing dieback of cocoa based on ITS sequence analysis

3.2. Introduction

Identification of fungi traditionally has relied upon visual examination of microscopic features, especially sporing structures, augmented by gross morphology of macroscopic features *in situ*, colony characteristics on artificial media, and biochemical reactions (Hawksworth, 1991; Sutton and Cundell, 2004). Such approaches have served well in the past but specialist knowledge is often needed to differentiate to species level in large and complex genera such as *Penicillium*, *Aspergillus*, and *Fusarium* (Larone, 1995; Henry *et al.*, 2000; Leslie and Summerell, 2006). The range of isolates described in Chapter 2 exhibit considerable morphological diversity, and for *Fusarium* the macroconidial dimensions frequently fell outside the range previously reported for *Fusarium decemcellulare* on cocoa (Leslie and Summerell, 2006). Given the desirability of accurate identification of isolates to be used when screening for resistance and the potential for mixed infections with multiple species there is a clear need for reliable identification of all species involved in dieback disease. The potential advantages of using molecular biology techniques for the diagnosis of a number of diseases have been widely discussed lately. In the last decade, numerous DNA-based methods have been developed to improve the diagnosis of fungal infections and the identification of plant pathogenic and other fungi (Gottfredsson *et al*., 1998; Walsh and Chanock, 1998). These methods are particularly promising because of their simplicity, specificity, sensitivity and potential for scaling up to handle large numbers of isolates. For

example, PCR methods targeting different genes have been described elsewhere for identification of *Cryptococcus neoformans* (Tanaka *et al*., 1996), *Aspergillus fumigatus* (Kobayashi *et al*., 1999; Skladny *et al*., 1999), and species of *Candida* (Hidalgo *et al.*, 2000). A number of studies have also described probes, restriction fragment length polymorphism, or other methods to identify unique ribosomal DNA (rDNA) sequences (Evertsson *et al*., 2000; Kauffman *et al.*, 2000; Loeffler *et al.*, 2000). The most common approaches have targeted portions of the fungal rDNA and sequence information on a wide range of fungi has accumulated in the public database (Braun *et al*., 2000). Even with the proviso of occasional mis-identification of deposited sequences, this represents an invaluable resource with which to compare unnamed isolates or to confirm identity of the already assigned name. This study presents results from PCR and ITS1-5.8S-ITS2 region sequence analysis of *Fusarium* and *Lasiodiplodia* isolates described in Chapter 2.

3.3. Materials and Methods

3.3.1. Fungal samples and preparation of genomic DNA

The samples evaluated here were the pathogenic isolates previously characterised by morphology in Chapter 2. For DNA extraction, a single colony of each culture was transferred to a PDA plate and grown for ten days at 30 °C followed by DNA isolation with the Qiagen DNeasy Plant Mini Kit (Qiagen Ltd., Qiagen House, Fleming Way, West Sussex, UK). Approximately 100 mg of macerated mycelium was transferred to a clean Eppendorff tube and 400 µl of Buffer AP1 and 4 µl of RNase-A were added followed by incubation at 65 °C for 10 minutes. Lysis Buffer AP2 (130 µl) was added and the lysate mixed gently by inverting and then incubated on ice for 5 minutes. The solution was centrifuged for 5 min at 13,000 × g and the supernatant transferred to a QIAshredder spin column tube. This was centrifuged for 2 min at 13,000 × g and the flow-through fraction transferred to a new tube without disturbing the pellet. Typically, 450 µl of lysate was recovered so one and a-half volumes of Buffer AP3/E (i.e. 675 µl) was added to the lysate and mixed by pipetting. Six hundred and fifty microlitres (650 µl) of the mixture including any precipitate,

were transferred to a DNeasy mini spin column tube and recentrifuged for 1 minute at 6,000 × *g*. The flow-through was discarded but the sediment in the collection tube was washed twice with 500 µl wash Buffer (AW) and centrifuged to dry the membrane of the column. Two successive elutions were performed with 100 µl of preheated (65 °C) Buffer AE placed directly onto the DNeasy membrane at room temperature. The eluate containing the DNA was pooled and stored at -20 ˚C until use. The presence of DNA was confirmed at this stage by running the samples on Tris-acetic acid-EDTA-agarose gel (0.8 %, w/v), and their concentrations determined using an automated Eppendorf BioPhotometer and 1:20 dilutions of each DNA sample (Figure 39).

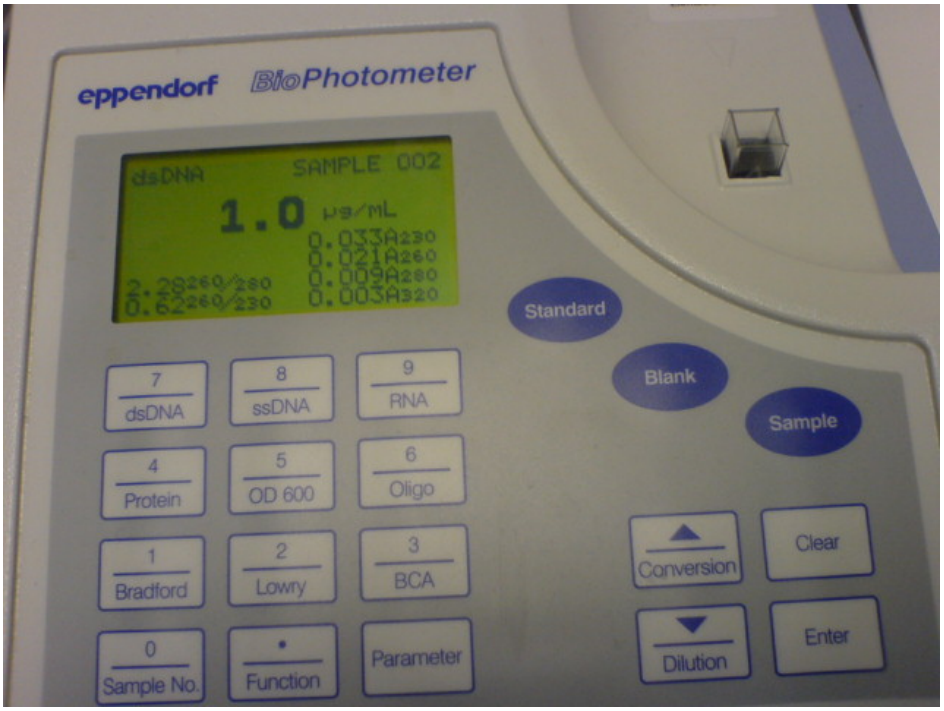

Figure 39. The Eppendorf BioPhotometer used for measuring DNA concentration.

3.3.2. PCR Amplification of Ribosomal DNA Regions

The internal transcribed spacer (ITS) fragment which includes the ITS1, the 5.8S rDNA gene and the ITS2 regions (Figure 40) was amplified using two primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) White *et al*. (1990). Each of the 25-µl PCR reaction mixtures consisted of 12 µl PCR

Biomix^{TM} Red (Bioline), 2.5 ng of template genomic DNA, 0.5 µl of each primer (10 pmol), 0.5 µl of $MgCl_2$ (50 mM), and 9 µl of DNase free water. Control amplifications using primers only were made to ensure that the reagents used were not contaminated with extraneous DNA. PCR reaction was conducted using a Whatman Biometra T1 Thermocycler according to the following protocol: initial denaturing at 95 ˚C for 5 minutes, 94 ˚C for 1 minute, annealing at 50 ˚C for 1 minute, and extension at 72 ˚C for 45 seconds. These cycles were repeated 35 times with a final step at 72 °C for 7 minutes, and then storage at 4 °C. The PCR products were detected by agarose gel [0.8 % (w/v)] electrophoresis and staining with GelRed^{TM} Nucleic Acid Gel Stain with subsequent visualisation and photography under UV transilluminator.

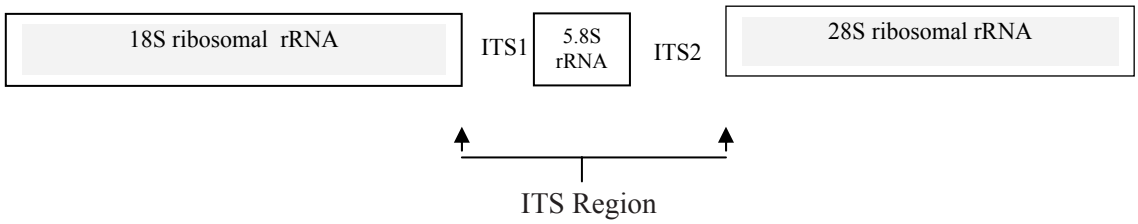

Figure 40. Diagrammatic representation of arrangement of ribosomal DNA including coding regions for the 5.8S and 28S rRNA and PCR primer binding sites. ITS = internal transcribed spacer region. Primers used to amplify single-stranded DNA for sequencing ("ITS1" and "ITS4") were designed and named by White *et al.* (1990).

3.3.3. PCR cycle sequencing reaction

PCR-generated DNAs as above, were purified from agarose gel using Genomics Montage Millipore gel extraction kit (Millipore Corporation, Bedford, MA 01730 USA) and directly sequenced in both forward and reverse directions using the Big Dye terminator system, version 3.1 (Applied Biosystems, Foster City, California) using an ABI 377 sequencer. The protocol per reaction done in a 96-well plate (10 µl reaction) is shown in Table 11. There were 35 PCR cycles and thereafter, 80 µl of absolute ethanol was added to each reaction well. The microtitre plate was incubated at room temperature for 15 minutes and centrifuged at ≈ 20 ˚C for 45 minutes at 4000

rpm. The supernatant was drained by blotting on tissue paper followed by addition of 150 µl of 70 % ethanol.

Table 11. Reagents and their quantities used per 10 µl in a 96-well plate.

Reagent	Quantity
Template DNA	2.0 µl
Primer (10 pmol)	1.0 µl
5X BigDye Buffer	1.75 µl
ABI BigDye (v3.1) Mix	0.5 µl
ddH_2O	4.75 µl

The sample was run in a Thermal Cycler (Uno II, Biometra) using the following steps:

Lid temperature - 105 °C

Step 1 - Incubation at 94 °C for 2 minutes

Step 2 – Incubation at 96 °C for 15 seconds

Step 3 – Incubation at 50 °C for 15 seconds

Step 4 – Incubation at 60 °C for 4 minutes

Step 5 – Repeated to step 1 for 35 times

Step 6 – Incubation at 10 °C hold

3.3.4. Sequence alignment and phylogenetic analysis

The sequence chromatograms were edited using Sequencher 4.8 software (Genecodes Corp., Ann Arbor, Michigan), and searches were carried out in BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the closest matches in the public database. The ITS sequences of *Fusarium* and *Lasiodiplodia* were manually aligned with MacClade 4.06 OSX. A likelihood bootstrap analysis was performed with 100 replications using RAxML 7.0.4 (Stamatakis *et al*., 2008).

3.4. Results

3.4.1. Amplification of ITS regions

The ITS sequences of all isolates were determined and their information deposited at GenBank that gave accession numbers FJ545330 to FJ545407 (Table 13). Two phylogenetic trees were constructed using RAxML for *Fusarium* and *Lasiodiplodia*. An assessment of the action of the primers on the extracted genomic DNA indicated that the primers amplified products of the test DNA samples, and resolved prominent bands of around 550 bp at the selected annealing temperature of 50 °C. Figure 41 shows PCR products (amplicons) of some of the fungal isolates on agarose gels. As expected, the universal primers produced amplicons that were identical and indistinguishable between the different isolates. The 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence of all isolates from Ghana and the CABI reference isolates (Chapter 2) were successfully amplified from DNA by the fungus-specific universal primer pair ITS1-ITS4. Alignment of sequences of all fungal DNAs required one or more gaps and occasionally nucleotides of unknown identity denoted by question marks. The variation (deletions or insertions) along the entire stretch of sequences was limited to a few bases in the isolates studied. The alignment of the *Fusarium* species showed that the stretches 1-106 (18S ribosomal RNA), 311-428 (5.8S ribosomal RNA) and 522 to >550 (28S ribosomal RNA) were highly conserved (less than 20 nucleotide substitutions) and that variability was mostly restricted to stretches 107-310 (internal transcribed spacer 1) and 429-511 (internal transcribed spacer 2). When comparing the *Lasiodiplodia* alignments with GenBank sequences, it was observed that the stretches 1-3 (18S ribosomal RNA), 140-298 (5.8S ribosomal RNA) and 462 to >480 (28S ribosomal RNA) were evolutionarily conserved and that variability was limited to stretches 4-139 (internal transcribed spacer 1) and 299-461 (internal transcribed spacer 2. Aligned DNA sequences of ITS1, the 5.8S subunit and ITS2 from five of the studied isolates are presented in (Table 12). The 18S ribosomal RNA region of the *Lasiodiplodia* species was shorter because rejecting bits off the ends of ambiguous

sequences during the alignment process may have trimmed away some sections of that region.

Table 12. DNA sequence information of two *Lasiodiplodia* and three *Fusarium* species.

L. pseudotheobromae AC008	ttaccgagttttcgggctt- ggtcgactctcccacccttttgtgaacgtacctctgttgctttggcggctccggccgccaaaggacctccaaactccagtcagtaaacgcagacgtctgataaaacaa gttaataaactaaaaactttcaacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagtaatgtgaattgcagaattcagtgaatcatcg aatctttgaacgcacattgcgccccttggtattccgggggggcatgcctgttcgagcgtcattacaaccctcaagctctgcttggaattgggcaccgtccatcctgcgg acgcgcctaaagacctcggcggtggctgttcagccctcaagcgtagtagaatacacctcgctttggagtggttggcgtcgcccgccggacgaaccttctgaactttt ctcaaggtagacctcggatcaggtagga-t-cccg [482 bp]
L. pseudotheobromae IMI333797	?????gagttttcgagctccggctcgactcttccacccttttgtgaacgtacctctgttgctttggcggctccggccgccaaaggacctccaaactccagtcagtaaac gcagacgtctgataaaacaagttaataaactaaaactttcaacaacggatctcttggttctggcatcgatgaagaacgcagcgaaatgcgataagtaatgtgaattg cagaattcagtgaatcatcgaatctttgaacgcacattgcgccccttggtattccgggggggcatgcctgttcgagcgtcattacaaccctcaagctctgcttggaattg ggcaccgtcctcactgcggacgcgcctcaaagacctcggcggtggctgttcagccctcaagcgtagtagaatacacctcgctttggagcggttggcgtcgcccgc cggacgaaccttctgaactttttctcaaggttgacctcggatcaggtaggaa-acccg [484 bp]
F. solani AC580	---------ttcctccgc-tttat--gatatgcttaagttcag-cg-ggtattcct-acctgattcgaggtca---ytt-cagaaagagttg-ggtgtttt- acggcgtggccgcgccgctctctccagtcgcgaggtgttagc-tactacgcgatggaagctgcggcgggaccgccactgtatttg-gggg-a-cggcgt-g-tg--- ccc-a-cgggggggct—ccgccgatccccaacgccaggcccgggggcctgagggttgtaatgacgctcgaacaggcatgcccgccagaa- tactggcgggcgcaatgtggcgttcaaagattcgatgattcactgaattctgcaattcacattacttatcgcatttcgctgcgttcttcatcgatgccagagccaagagaga tccgttgttgaaagttttaatttatttgc-ttgttttactcagaagaaacattatagaaa- cagagttaagggtcctctggcgggggcccgtttcacggggccgtctattcccgccgaagcaacgtttaggtagttcacagggttgatgagtgaaaactcggtaatg atccctccgcaggttcacctacgga--- [563 bp]
F. decemcellulare IMI361352	--------tttcctccgc-tttat—gatatgcttaagttcagtcgcggtattcct-acctgatccgaggtcaa-catt-caga—agttgggggggttt- aacggcttggccgcgccgcgttccagttgcgaggtgttagc-tactacgcaatggaggctacagcgagaccgccactagatttg-gggg-a-c—---ggc-g— a-ctatc-gc--- cgatccccaacaccaagccctagggcttgagggttgaaatgacgctcgaacaggcatgcccgccagaatactggcgggcgcaatgtgcgttcaaagattcgat gattcactgaattctgcaattcacattacttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgttgttgaaagttttgatttatttgttttgttttactcag aagatccacaagaatacatagagtttggggttcctctggcagcgagcggcgcccgatttctcggagcaccgtcgttgagtctgccgaggcaaattataggtatgttc acagggtttgggagttgtaaactcggaagtgatccctccgcagtcc---------- [558 bp]
F. chlamydosporum AC174	----------tcctccgc—ttatt-gatatgcttaagttcag-cg-ggtattcct-acctgatccgaggtcaa-catt-caga—agttg—gggtttt- acggcgtggccgcgacgattaccagtaacgaggtgtatgattactacgctatggaagctcga-cgtgaccgccaatcgatttg-ggg-aa-c------gc-ggg—t- t-a—cc-g-cgagtcccaacaccaagc--- tgagcttgagggttgaaatgacgctcgaacaggcatgcccgccagaatactggcgggcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaattcac attacttatcgcattttgctgcgttcttcatcgatgccagaaccaagagatccgttgttgaaagttttgatttatttgtttgttttactcagaagttccactaaaaacagagtttta ggg-tcctcgggccgtcccgttttacggggcgggctgatccgccgaggcaacgtataggtatgttcac-agggg-tttggg- agttgtaaactcggtaatgatccctccgcaggtccacctacgg---------- [544 bp]

Each sequence contains coding sequence for 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA. Sequence symbols: a, c, g, t = dATP, dCTP, dGTP, dTTP, respectively; r = a or g; y = c or t; hyphens = gaps; question marks = missing nucleotides or nucleotides of unknown identity.

Table 13. List of *Fusarium* and *Lasiodiplodia* species (isolated from diseased cocoa trees) identified by ITS sequence analysis.

Fungus species	Isolate number	Base pair length	GenBank accession no.
F. decemcellulare	IMI 380504	551	FJ545369
F. decemcellulare	IMI 361352	558	FJ545370
F. chlamydosporum	AC 037	480	FJ545407
F. chlamydosporum	AC 110	550	FJ545384
F. chlamydosporum	AC 120	471	FJ545385
F. chlamydosporum	AC 174	544	FJ545386
F. chlamydosporum	AC 197	486	FJ545387
F. chlamydosporum	AC 206	473	FJ545388
F. chlamydosporum	AC 229	477	FJ545389
F. chlamydosporum	AC 255	544	FJ545390
F. chlamydosporum	AC 276	542	FJ545391
F. chlamydosporum	AC 284	545	FJ545392
F. chlamydosporum	AC 328	535	FJ545393
F. chlamydosporum	AC 330	505	FJ545394
F. chlamydosporum	AC 402	533	FJ545395
F. chlamydosporum	AC 451	512	FJ545396
F. chlamydosporum	AC 593	547	FJ545398
F. chlamydosporum	AC 638	543	FJ545399
F. chlamydosporum	AC 656	547	FJ545400
F. chlamydosporum	AC 705	546	FJ545401
F. chlamydosporum	AC 773	546	FJ545402
F. chlamydosporum	AC 914	546	FJ545405
F. oxysporum	AC 317	477	FJ545406
F. oxysporum	AC 511	511	FJ545397
F. oxysporum	AC 787	512	FJ545403
F. oxysporum	AC 806	516	FJ545404
F. proliferatum	AC 031	559	FJ545371
F. proliferatum	AC 392	549	FJ545376
F. proliferatum	AC 748	521	FJ545380
F. proliferatum	AC 767	478	FJ545381
F. solani	AC 076	493	FJ545372
F. solani	AC 211	515	FJ545374
F. solani	AC 219	565	FJ545383
F. solani	AC 361	521	FJ545375
F. solani	AC 551	565	FJ545377
F. solani	AC 580	563	FJ545378
F. solani	AC 739	510	FJ545379
F. solani	AC 768	566	FJ545382
F. solani	AC 995	505	FJ545373
L. pseudotheobromae	IMI 333797	484	FJ545364
L. pseudotheobromae	AC 008	482	FJ545331
L. pseudotheobromae	AC 036	482	FJ545332
L. pseudotheobromae	AC 064	485	FJ545333
L. pseudotheobromae	AC 068	484	FJ545334
L. pseudotheobromae	AC 280	485	FJ545336
L. pseudotheobromae	AC 318	483	FJ545337
L. pseudotheobromae	AC 322	484	FJ545338
L. pseudotheobromae	AC 329	484	FJ545339
L. pseudotheobromae	AC 360	485	FJ545340
L. pseudotheobromae	AC 371	484	FJ545341
L. pseudotheobromae	AC 375	484	FJ545342
L. pseudotheobromae	AC 383	483	FJ545343
L. pseudotheobromae	AC 407	484	FJ545344
L. pseudotheobromae	AC 450	484	FJ545346
L. pseudotheobromae	AC 492	485	FJ545366
L. pseudotheobromae	AC 536	485	FJ545330
L. pseudotheobromae	AC 564	484	FJ545351
L. pseudotheobromae	AC 568	484	FJ545352
L. pseudotheobromae	AC 581	484	FJ545353
L. pseudotheobromae	AC 640	485	FJ545355
L. pseudotheobromae	AC 644	484	FJ545356
L. pseudotheobromae	AC 680	485	FJ545358
L. pseudotheobromae	AC 742	485	FJ545360
L. pseudotheobromae	AC 810	485	FJ545361
L. pseudotheobromae	AC 845	485	FJ545365
L. pseudotheobromae	AC 857	485	FJ545362
L. pseudotheobromae	AC 972	484	FJ545363
L. theobromae	AC 220	481	FJ545335
L. theobromae	AC 420	482	FJ545345
L. theobromae	AC 456	484	FJ545347
L. theobromae	AC 487	485	FJ545348
L. theobromae	AC 496	485	FJ545349
L. theobromae	AC523	484	FJ545350
L. theobromae	AC 639	483	FJ545354
L. theobromae	AC 673	483	FJ545357
L. theobromae	AC 718	481	FJ545359

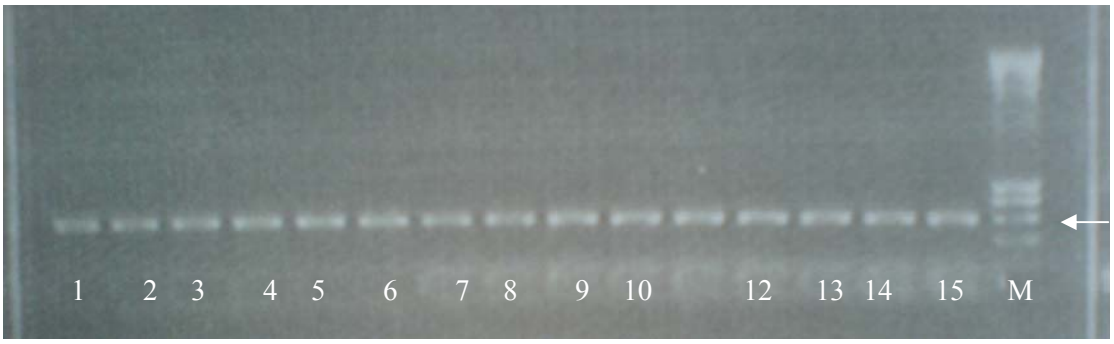

Figure 41. Amplification products of some *Fusarium* and *Lasiodiplodia* isolates by primers ITS 1 and ITS 4; lane M, 100-bp DNA length ladder; (hyper ladder iv); lane 1, isolate IMI 380504; lane 2, isolate IMI 361352; lane 3, isolate AC 995; lane 4, isolate AC806; lane 5, isolate AC 511; lane 6, isolate AC 551; lane 7, isolate AC 638; lane 8, isolate, 773; lane 9, isolate IMI 333797, lane 10, AC 492; lane 11, AC 845; lane 12, AC 972; lane 13, AC 857; lane 14, AC 680; lane 15, AC 810.

3.4.2. Identification of *Fusarium* isolates by ITS sequence analysis.

The 39 isolated dieback-associated fungal strains (AC037-AC995) (Table 13) were previously listed as 37 unidentified *Fusarium* spp. and two *F. decemcellulare* isolates by means of colony and macroconidia morphology (Table 4 and Table 6). The two latter isolates were confirmed as *Nectria rigidiuscula* (anamorph *F. decemcellulare*) by likelihood cladogram analysis in the current study. The ITS sequence of the remaining 37 isolates showed close matches to the ITS sequence of four other *Fusarium* species namely, *F. chlamydosporum*, *F. oxysporum, F. proliferatum* and *F. solani* (Table 13). Together with *F. decemcellulare*, the phylogenetic tree (Figure 42) divided them into four clades: one clade consisted of nine isolates of *F. solani* and the two reference isolates of *F. decemcellulare*. In this clade, *F. decemcellulare* was derived first and *F. solani* later. The total of eleven species included here formed a monophyletic group. The homology of nucleotide sequences among members of this clade was significant with between 56 and 100 % bootstrap support. The *F. decemcellulare* isolates 380504 and IMI362352 presented identical profiles and were grouped at a 100 % similarity level. The isolates *F. solani* AC076, *F. solani* AC211, *F. solani* AC219, *F. solani* AC361, *F solani* AC551, *F. solani* AC580, *F. solani* AC739, *F. solani* AC768, and *F. solani* AC995, grouped into a clade at 74 % with the reference *F. decemcellulare* isolates from CABI.

The general phylogeny of the *Fusarium* species analysed is given in Figure 42. None of the *Fusarium* isolates from the diseased cocoa stems was tightly linked to *F. decemcellulare* although *F. solani* isolates showed the closest relatedness. The isolates of *Fusarium solani* and *Fusarium decemcellulare* represented two similar branches within clade 1 of the *Fusarium* species with 75 % bootstrap support to the other isolates. A BLAST search revealed that the *F. oxysporum* isolates used in this study had their ITS sequences matching closely with those from the GenBank (GenBank accession no. AY928409). The output of the BLAST search of the ITS sequence of *F. decemcellulare* IMI380504 and *F. decemcellulare* IMI362352 showed 99 % sequence identity with *Nectria rigidiuscula* (GenBank accession no. FJ478113). Likewise searches of the sequences of *F. solani* isolates matched the ITS sequence (99 % identity) of reference *F. solani* (GenBank accession no. FJ478128), the *F. chlamydosporum* isolates had 99 % sequence identify with reference *F. chlamydosporum* (GenBank accession no. DQ489296), and *F. proliferatum* isolates were 99 % identical with *F. proliferatum* (GenBank accession no. EU151487).

The second major clade from the cladogram further divided into two sub-clades; one cluster consisting of *F. oxysporum*, and the other of *F. chlamydosporum.* However, there was no bootstrap support for these clusters in the data (bootstrap value <50 %). The last clade containing *F. proliferatum* was well defined and its monophyly supported to 95 % in bootstrap value. This group was less closely related to the other *Fusarium* species and it is noteworthy that all *F. proliferatum* species were collected from the same source (Table 4, Chapter 2). *Fusarium proliferatum* species formed a sister-group with *Fusarium chlamydosporum* but with a low bootstrap support value. The ITS sequences has thus permitted distinguishing the existence of four species of *Fusarium* attacking cocoa in Ghana other than *F. decemcellulare.* However, no more conclusions could be made as only isolates from the Eastern and Western Regions were used in the study.

3.4.3. Identification of *Lasiodiplodia* isolates by ITS sequence analysis.

A total of 37 field isolates, including one reference CABI isolate, IMI333797 were analysed. Results of phylogenetic analysis using MacClade alignment and RAxML tree construction supported the results of BLAST analyses of the ITS sequences. The BLAST search using matching sequences in the GenBank database indicated that all *Lasiodiplodia* species sequences were closely related to each other and belonged to either *L. theobromae* or *L. pseudotheobromae*. Twenty-seven of these isolates most consistently aligned with *L. pseudotheobromae* with up to 99 % sequence identity whilst the rest were classified as *L. theobromae* (Table 13). *Lasiodiplodia* sequences varied at several positions but they remained more related to each other than to any other species. Because all samples were most closely related to these two species it suggests that other *Lasiodiplodia* infections were not present in the dieback-diseased plants from the area surveyed. Figure 43 shows comparisons of the ITS sequence relatedness of the isolates. The bootstrap values supporting the clades of *Lasiodiplodia* sequences ranged from about 63 to 72 % and many had low values less < 50 %. This suggests that there may have been variation in the different sequences that were not accounted for by the cladogram. All isolates clustered into five clades. One clade contained the following isolates *L. pseudotheobromae* AC008, *L. pseudotheobromae* AC280, *L. pseudotheobromae* AC360, *L. pseudotheobromae* AC008, *L. pseudotheobromae* AC375, *L. pseudotheobromae* AC383, *L. pseudotheobromae* AC450, *L. pseudotheobromae* AC536, *L. theobromae* AC639, *L. theobromae* AC718, *L. pseudotheobromae* AC845 and *L. pseudotheobromae* IMI333797 while the isolates *L. theobromae* AC220, *L. theobromae* AC420, *L. theobromae* AC456, *L. theobromae* AC487, *L. theobromae* AC496, *L. theobromae* AC523 and *L. theobromae* AC673 belonged to another clade. The following isolates, *L. pseudotheobromae* AC064, *L. pseudotheobromae* AC318, *L. pseudotheobromae* AC322, *L. pseudotheobromae* AC329, *L. pseudotheobromae* AC492, *L. pseudotheobromae* AC568, *L. pseudotheobromae* AC581, *L. pseudotheobromae* AC640, *L. pseudotheobromae* AC644, *L. pseudotheobromae* AC680 and *L. pseudotheobromae* AC810 all clustered well into a separate clade while the two

remaining isolates, *L. pseudotheobromae* AC036 and *L. pseudotheobromae* AC407 each constituted a separate clade on its own. Despite CABI isolate IMI333797 being supplied as *L. theobromae* it was found to be more closely related to isolates of *L. pseudotheobromae* than to any of the other *L. theobromae*. BLAST analysis of its ITS sequence suggested that IMI333797 most likely belongs to *L. pseudotheobromae* because the degree of nucleotide sequence identity between it and the closest *L. theobromae* species relative was low whereas the match with *L. pseudotheobromae* ITS segments was closer. The output of the BLAST search of the ITS sequence of isolate IMI333797 and *Lasiodiplodia* isolate AC220 showed 99 % sequence identity with *L. pseudotheobromae* (GenBank accession no. EF622081) and *Botryosphaeria rhodina* (anamorph: *L. theobromae*) (GenBank accession no. EU600925), respectively.

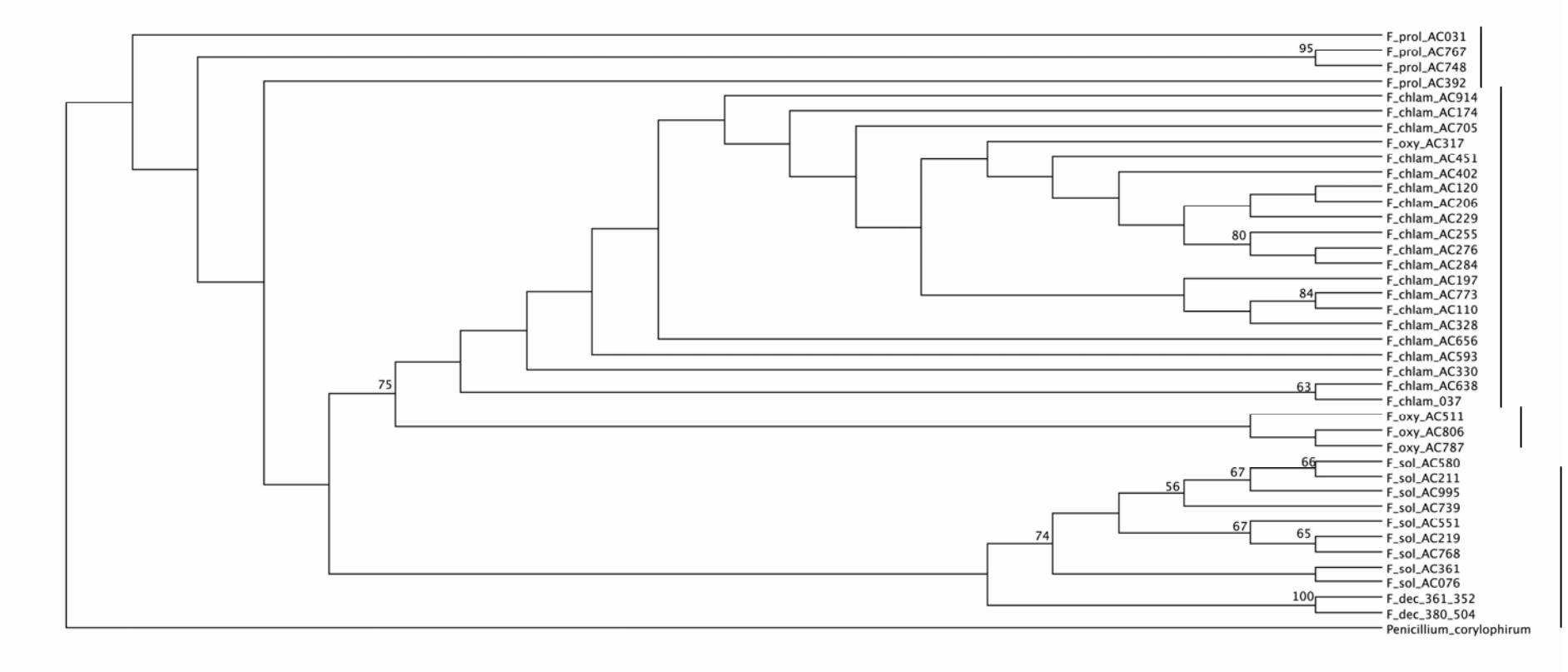

Figure 42. Cladogram revealing the relatedness of isolates of *Fusarium* based on 400 – 500 nucleotides from ITS1 and ITS2 rDNA. The DNA sequences were aligned with MacClade and trees were constructed with RAxML. The tree was rooted with *Penicillium corylophilum*. Numbers given on branches are bootstrap values indicating the confidence level from a 100-replicate bootstrap sampling. (Frequencies below 50% are not included).

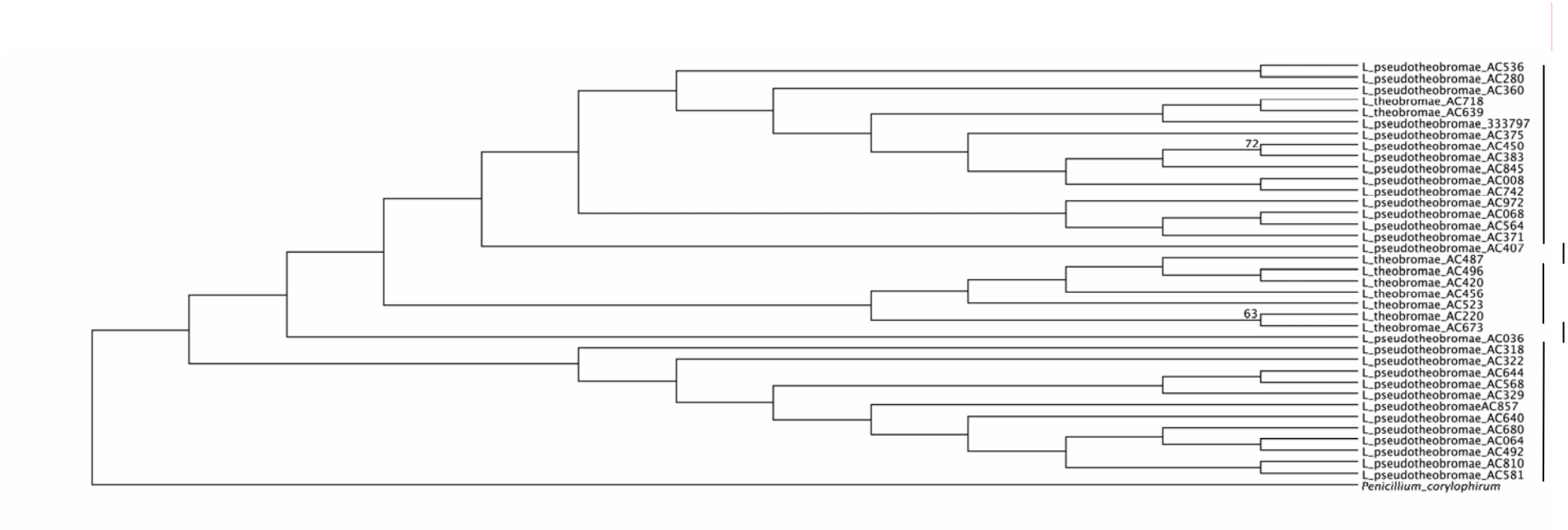

Figure 43. Cladogram revealing the relatedness of isolates of *Lasiodiplodia* based on 400 - 500 nucleotides from ITS1 and ITS2 rDNA. The tree was rooted with *Penicillium corylophilum.* Numbers given on branches indicate the confidence level from 100-replicate bootstrap sampling. (Frequencies below 50% are not included).

3.5. Discussion

A few fungi have been recognized as important stem canker pathogens of cocoa in West Africa, the most well known being *Fusarium decemcellulare* and *Lasiodiplodia theobromae* that have historically been isolated from diseased trees (Cotterell, 1927; Crowdy, 1947; Owen, 1956). The current observations (Chapter 2) indicated, however, that other *Fusarium* spp. could cause dieback disease in the sub-region. The true identities of these potential pathogens were unknown which partly prompted this study with the view to developing control measures for dieback disease. There was an obvious variation of pathogenicity rates across these isolates (Chapter 2) and this was probably due to differences inherent in the fungal populations and the geographical areas from where they were collected.

Traditional characterisation studies apply mainly morphological and cultural methods, but these tend to be time consuming and often provide equivocal results with what are widely regarded as 'difficult' genera such as *Fusarium.* Very few studies have been carried out on canker pathogens of cocoa and only a minority have involved molecular methods. Molecular techniques have been applied widely in ecological studies (Wirgin *et al*., 1997) and wildlife conservation (Avise and Hamrick, 1996) in the past several years. In many studies, the polymerase chain reaction (PCR) has proved powerful in detecting obscure genetic variation; for instance, in the DNA of symbionts that cannot be cultured and separated from their co-symbionts, and in the microbial floristic composition (Haddad *et al*., 1995; Hansen and Hanson, 1996). For systematic and ecological studies, sets of universal primers have been designed, which may be used across a range of taxa (Dumolin-Lapegue *et al*., 1997; Chiang *et al*., 1998; Chow and Hazama, 1998). The primer sequences of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA have been widely used for resolving phylogenetic relationships at the species or generic levels (White *et al*., 1990; Baldwin, 1992). Previous reports have documented the amplification of fungal sequences from plant foliage (Liston *et al*., 1996; Klein and Smith, 1996); and employing phylogenetic analysis, Camacho *et al*. (1997) was able to identify the endophytic fungi in *Picea* based on the ITS nucleotide sequences.

The current study presents a phylogenetic analysis of a few dozens of isolates obtained from diseased cocoa that were morphologically identified to the genera *Fusarium* and *Lasiodiplodia*. A blast search of the rDNA genome sequence defined by the universal fungal primers ITS1 and ITS4 suggested that some of the pathogenic isolates are *F. solani* while others belong to the species *F. chlamydosporum*, *F. oxysporum* and *F. proliferatum* (sexual stage *Gibberella intermedia*, O'Donnell *et al*., 1998). The sequence data provided evidence that *Fusarium* isolates from cocoa and the two reference isolates from CABI (*F. decemcellulare* IMI380504 and IMI362352) were separate species. *Fusarium decemcellulare* surprisingly had very limited information in the public database probably due to little work that has been carried out on the fungus. Numerous challenges exist regarding the identification of *Fusarium* spp. and several taxonomic systems have been proposed (Booth, 1971; Matuo, 1980; Nelson *et al*., 1983; Booth and Sutton, 1984; Leslie and Summerell, 2006). For example, the isolate named as *F. fusarioides* in Booth's system (Booth, 1971) was later referred to as *F. chlamydosporum* in the system of Nelson *et al*. (1983) whereas the fungus named as *F. roseum* and *F. semitectum*, by Booth (Booth, 1971) and Matuo and Snyder (1973), respectively was later named *Fusarium pallidoroseum* (Booth and Sutton, 1984). What was remarkable in the current study was that, contrary to prior expectation, none of the *Fusarium* isolates from cocoa was *F. decemcellulare.*

Blast search and likelihood analysis of the ITS sequences of the *Fusarium* species from cocoa showed that there were distinct clades although many of the isolates are very closely related. This analysis supports the distinct position of the species *Fusarium decemcellulare*. In a comparison of the ITS sequences, the study found that *F. decemcellulare* and *F. solani* formed a monophyletic group which was distinct from separate clades comprising each of *F. oxysporum, F. chlamydosporum*, and *F. proliferatum*. Culturally all species looked similar and the symptoms caused were indistinguishable in appearance on cocoa (Chapter 2), although the disease developed somewhat slower with the isolates of *F. oxysporum* and *F. proliferatum* than the others. *Fusarium oxysporum* is responsible for an enormous range of plant diseases, usually involving a vascular wilt syndrome (Leslie and Summerell, 2006). The majority of *F. oxysporum* isolates causing vascular wilts are specific strains that infect only a small number of host plants, and have been differentiated from each other

using the sub-specific term forma specialis (f. sp.). For example, the strains commonly attacking banana are assigned to *F. oxysporum* f. sp. *cubense*; cotton, *F. oxysporum* f. sp. *vasinfectum*; and tomato, *F. oxysporum* f. sp. *lycopersici*. Morphologically these strains are identical, and they also cannot be differentiated from non-pathogenic or saprophytic strains, of which there is a huge diversity, especially in soil. From a diagnostic point of view, the separation of this species into formae speciales has important diagnostic and quarantine implications. Identification of these strains has traditionally involved pathogenicity testing with sets of host differentials appropriate for the formae speciales in question. However, these tests are time-consuming to set up and can require long periods of time, for example, four to six months for *F. oxysporum* f. sp. *canariensis*, before they could be scored definitively. To add to the confusion there are reports (Jiménez-Gasco *et al.*, 2002) that formae speciales are not monophyletic, with pathogenicity to plant species arising independently on multiple occasions from different genetic backgrounds. The relationship of *F. oxysporum* isolates detected here from cocoa to the species as a whole must await further study.

While status of *F. solani* and *F. oxysporum* as plant pathogens is well established, *F. chlamydosporum* and *F. proliferatum* are hitherto regarded as relatively insignificant pathogens. On sunflower they are reported as causing collar rot, seedling rot, wilting and tip burning (Sharfun-Nahar and Mushtaq, 2007), but otherwise there are few records of pathogenesis especially on woody hosts.

The BLAST search using matching sequences in the GenBank database indicated that all *Lasiodiplodia* species' sequences were most closely related to either *L. theobromae* or *L. pseudotheobromae*. Based on the ITS phylogeny obtained in the present study (Figure 43), five clades could be recognised for these species that have anamorphs related to the *Botryosphaeriaceae*. Twenty-seven of these isolates most consistently aligned with *L. pseudotheobromae* with up to 99 % sequence homology whilst the rest were classified as *L. theobromae.* A CABI reference *Lasiodiplodia* isolate (IMI333797) that originally was identified as *L. theobromae* isolated from cassava in Nigeria, phylogenetically was linked to the *L. pseudotheobromae* group. Conidia of these two species are similar, and the CABI isolate was identified as *L. theobromae* because *L. pseudotheobromae* was differentiated only in 2008 by Alves *et al*.

Very recently, it has been shown that *Botryosphaeria* comprises several different phylogenetic lineages (Alves *et al.*, 2008) that correlate well with morphological features of the anamorphs but observable morphological differences were lacking in the isolates studied in Chapter 2. The studies by Phillips *et al.* (2006) suggested *Botryosphaeria* constitutes a relatively small genus consisting of only *B. dothidea* (Moug. Fr.) Ces. & De Not. (the type species of the genus) and *B. corticis* (a species restricted to *Vaccinium* spp.). The remaining lineages within what was known as *Botryosphaeria* now consist of the anamorph genera *Diplodia* (including *Sphaeropsis*), *Lasiodiplodia*, *Neofusicoccum*, *Pseudofusicoccum*, *Macrophomina*, *Neoscytalidium* and *Dothiorella* (Crous *et al*., 2006). It was recently determined that *Diplodia seriata* De Not. was the correct name for the anamorph of "*Botryosphaeria*" *obtusa* (Phillips *et al*., 2007).

The type species *L. theobromae*, geographically widespread in the tropics and subtropics, has been associated with approximately 500 hosts (Punithalingam, 1980). This apparently unspecialized plant pathogen has been reported to cause numerous diseases, including dieback, root rot, fruit rots, leaf spot and witches' broom amongst many others (Punithalingam, 1980). It is also said to occur as an endophyte (Mohali *et al*., 2005; Rubini *et al*., 2005). Less frequently it has been associated with keratomycosis and phaeohyphomycosis in humans (Punithalingam, 1976; Rebell and Forster, 1976; Summerbell *et al*., 2004). In view of its widespread occurrence, the large number of hosts and its known morphological variability (Punithalingam, 1980) it is suggested that *L. theobromae* might be composed of a number of cryptic species. Recently, Pavlic *et al*. (2004) described a new species *L. gonubiensis* Pavlic, Slippers & M. J. Wingf. on the basis of conidial morphology and dimensions, and ITS sequence data. In a similar way, Burgess *et al.* (2006) described three new *Lasiodiplodia* species (*L. crassispora*, *L. venezuelensis* and *L. rubropurpurea*) from the tropics on the basis of their ITS and EF1-α sequence data and morphological characters. *Lasiodiplodia pseudotheobromae* is another such cryptic species but based on the phylogeny of Figure 43 the robustness of the separation into two distinct species must be in doubt. Sequence analysis of a single region of the genome does not in itself provide a definitive phylogeny and support is required from other methods of assessing diversity, for example AFLP or RAPD analysis before the existence of a second species can be definitely accepted or rejected. In Pakistan, Khanzada *et al.*

(2004) reported that mango decline was a serious problem and of the pathogens, *L. theobromae* was the most abundant fungus while *F. solani* was the second most frequently isolated. The *Lasiodiplodia* isolates used in the present study are all pathogenic to cocoa (Chapter 2) but their ability to cross-infect other hosts is unknown. The natural history of the isolates in West Africa and the extent of their pathogenic potential must await further study.

4. Chapter 4

4.1. Resistance to canker and dieback in cocoa germplasm

4.2. Introduction

Ghana is noted for growing several plantation crops, of which the most important are cocoa, oil palm, coffee, cotton, and rubber. The main export crop, cocoa, generates some 30 to 40 % of foreign exchange earnings for the Ghanaian economy, and in 2005/2006, the country ranked second in dry cocoa bean production among the world's leading cocoa producing countries (Table 1; Chapter 1). However, losses of up to 30 % of potential yield of the crop occur every year because of dieback disease, which has symptoms developed from the action of *Fusarium decemcellulare*, *Lasiodiplodia theobromae* and some other *Fusarium* species. The Government of Ghana spends several million Ghana-cedis to purchase insecticides every year for use against mirids, the insects that provide entry points for the aforementioned pathogenic fungi.

After nearly 100 years experience of controlling dieback disease, the ideal control regimen is still to be found. The recent past years have seen problems with insecticide use and misuse (particularly in rural areas where there is the potential for contamination of ground water supply): high pesticide residues and the destruction of natural ecosystems have attracted much attention in the cocoa industry (Padi and Owusu, 2001). This has especially been the case since the introduction of new insecticides from Western countries got into full swing. Moreover, there is a worldwide demand for a reduction in the use of chemicals in agriculture and, therefore, a need to find economic, social and environmentally sound alternatives. Consequently, a number of other control approaches against mirids have been tried, but none against the fungal pathogens; however there are no effective or practical systems ready for adoption by cocoa farmers.

Few studies have addressed the effects of host resistance on dieback disease control in cocoa. In Ghana, Owen (1956) observed differences in susceptibility among different cocoa types to *F. decemcellulare*, but his collection did not consistently demonstrate worthwhile resistance. Although he suggested further testing of the Amelonado and Upper Amazon cocoa types, these genotypes, which have been grown for over half a century in West Africa, have continuously suffered severe damage.

Since many cocoa farmers are reluctant to rely on insecticides, the potential for alternative and sustainable control methods needed to be investigated. Moreover, interest has recently turned towards the production of organic cocoa because of increasing demand from import/export markets. Therefore, the availability of improved and dieback-resistant clones would be beneficial since it could combine vigour, high yield, and early maturity with reduction in pesticide use so that cocoa farmers can make substantial gains in their net income. In Owen's work resistance was assessed using lesion size but in the current study, a range of different independent factors namely, incubation period, lesion expansion, and foliar dieback incidence were also used.

4.3. Materials and methods

4.3.1. Resistance screening

Resistance of cocoa clones to infection was studied in two different experiments: 1. artificial inoculation in the laboratory on detached stems. 2. Greenhouse *in-vivo* inoculation of vegetatively propagated cocoa clones.

4.3.2. Experiment 1

4.3.2.1. Clones and fungal isolates

Clones screened in the laboratory are listed in Table 14; fan branches of 29 accessions of cocoa germplasm with similar vigour were collected from ten-year-old plants growing in the field Unit of the International Cocoa Quarantine Centre (ICQC) based at the University of Reading, UK. Approximately 5 cm stem segments [diameter 4 to 5 mm] (four replicates each) were cut from the detached branches. They were washed with distilled water and surface sterilized for 10 seconds in a 0.5 % sodium hypochlorite solution, followed by four one-minute rinses in sterile distilled water. The control consisted of Amelonado, which is susceptible to dieback. All stem segments were maintained at room temperature (23 to 27 °C) with 12 h of light per day under fluorescent tubes two metres above. Two *Lasiodiplodia* isolates designated AC371 and AC845 and one isolate of *Fusarium decemcellulare* IMI380504 (Table 15) obtained from diseased plants (Chapter 2) were selected for the detached stem *in-vitro* assay. Isolates AC371 and AC845 were collected as part of the current study while the third was originally isolated from a *Ceiba* tree by CABI, Biosciences, UK. The three isolates were chosen for this study based on their previously determined differences in greenhouse pathogenicity tests as discussed in Chapter 2.

The fungal inocula were maintained as pure cultures *in-vitro* on PDA at 4 °C in the laboratory at Silwood Park. Prior to inoculations, all cultures were transferred from storage onto new PDA plates and incubated under a 14-hour light: 10-hour dark photoperiod at 30 °C to allow renewed growth and conidia formation. From these actively growing cultures, conidia were collected and suspended in distilled water for inoculum preparation following the method of Stack (1989) with the resultant spore suspension adjusted to 1×10^6 conidia per ml using a haemocytometer.

Table 14. List of cocoa germplasm (including their donor GenBank and GenBank accession numbers) used in the laboratory detached stem inoculation.

Clone	Donor GenBank	Reading accession number
AMAZ 15/15	Kew, UK	RUQ 1
AMAZ 3/2	ICG, T	RUQ 91
AMAZ 5/2	ICG, T	RUQ 93
AMELONADO	-	-
BE 2	CATIE	RUQ 117
CATIE 1000	CIRAD-CP	RUQ 844
CRU 100	ICG, T	RUQ 877
CRU 124	-	RUQ-
EET 272	Kew, UK	RUQ 6
EET 59	CIRAD-CP	RUQ 845
GU 123/V	CIRAD-CP	RUQ 1068
GU 125/C	CIRAD-CP	RUQ 190
GU 136/H	CIRAD-CP	RUQ 221
ICS 43	ICG, T	RUQ 144
LCTEEN 37/F	USDA, Miami	RUQ 153
LCTEEN 37/G	USDA, Miami	RUQ 154
LCTEEN 37/I	USDA, Miami	RUQ 156
MXC 67	ICG, T	RUQ 104
NA 149	ICG, T	RUQ 801
PA 107	Kew, UK	RUQ 35
PA 120	CIRAD-CP	RUQ 852
PA 137	Kew, UK	RUQ 36
PA 7	SABAH	RUQ 113
POUND 7/B	Kew, UK	RUQ 24
SC 20	ICG, T	RUQ 690
SC 9	ICG, T	RUQ 1064
SCA 6	ICG, T	RUQ 234
T85/799	CIRAD-CP	RUQ 855
UF 677	ICG, T	RUQ 346

Table 15. *Fusarium decemcellulare, *L. pseudotheobromae*$^{++}$ and *L. theobromae*$^{+}$, relative aggressiveness, host and origin.**

Isolate	Relative pathogenicity[1]	Host[2]	Origin[3]	Source
IMI380504*	Intermediate	*Ceiba*	Ghana	CABI, UK
AC371^{++}	High	Cocoa	Ghana	Silwood Park isolation
AC845^{+}	Low	Cocoa	Ghana	"

[1]Pathogenicity of isolates was determined using the mycelium-plug inoculation method on Amelonado cocoa.
[2]Host from which each isolate was taken.
[3]Location from where the diseased plant was originally taken.

Inoculation was done by making several punctures on the surface of each detached stem segment and then immediately the wounded end of the stem was dipped into a fungal spore suspension in 0.025 % Tween-20. One inoculation was given to each stem. They were marked, and then incubated vertically in humid plastic chambers lined with moist paper towels (Figure 44). The lesion length was recorded with a calliper 14 days after inoculation, and the lower portion of the stem sampled for fungal isolation. Prior to sampling, the epidermis was removed and the stem cut transversely open with a sharp, sterile scalpel. Small wood sections were cut out at different lengths below the point of inoculation and plated on PDA to obtain the fungal colonisation profile within the stem. A clone was considered tolerant if it showed necrosis close to the site of inoculation (≤ 2 mm), while susceptible genotypes were those with browning > 4 mm from the point of inoculation, recognised by the presence of dark brown lesions and viable mycelium when cultured on PDA. The experiment was repeated once and it was arranged in a split plot design with four replicates. Each stem segment was an experimental unit with isolates as the main plot factor and cocoa germplasm randomised within isolates as subplots.

Figure 44. *In-vitro* stem-segment inoculation using spore suspension near the top of the cuttings.

4.3.3. Experiment 2

4.3.3.1. Greenhouse screening of cocoa germplasm

In the greenhouse, Amelonado cocoa seeds were germinated, transplanted, and grown in a 2: 2: 1 mixture of multi-purpose compost, loam, and sharp sand in 17 cm deep × 18 cm diameter, ≈ 1.5 L capacity plastic pots. Fifteen clones (including Amelonado) were used in the greenhouse because some clones used in the laboratory could not provide suitable scion for use in the greenhouse. The fourteen clones (Table 16) obtained from the ICQC, were vegetatively propagated using the T-budding technique. Budwood cuttings were budded (grafted) on two to three week old Amelonado seedling rootstocks. The stem of a seedling was cut just deeply enough to slice the bark and make a T-shaped incision two to three centimetres long five to ten centimetres from the soil. The flaps of the 'T' were opened outwards to reveal the cambium layer and a shield of bud was sliced out smoothly including a thin layer of the wood from the budwood. The bud was inserted into the slit made on the rootstock until it was even with the cross cut. Once set in place, the cut was pulled together by winding a 10 to 12 cm long budding rubber around the stem to hold the flaps tightly over the bud to prevent drying. The budded clones were grown in the greenhouse (20 to 30 °C) (Figure 21; Chapter 2). Eight to ten pots were raised for each of the 14 clones but not all groups had the full complement of eight to ten due to the failure of some buds to attach successfully. Consequently, five replicates of each clone were used for the tests. Amelonado variety was included as a susceptible check for comparison.

Table 16. List of cocoa germplasm used in the greenhouse, including donor GenBank and GenBank accession numbers.

Clone	Donor GenBank	Reading accession number
AMELONADO	-	-
CATIE 1000	CIRAD-CP	RUQ 844
EET 59	CIRAD-CP	RUQ 845
ICS 1	CIRAD-CP	RUQ 847
ICS 95	CIRAD-CP	RUQ 1144
IMC 67	ICG, T	RUQ 1056
LCTEEN 37/A	USDA, MIAMI	RUQ 148
LCTEEN 37/F	USDA, MIAMI	RUQ 153
LCTEEN 37/G	USDA, MIAMI	RUQ 154
MXC 67	ICG, T	RUQ 104
PA 7 (PER)	SABAH	RUQ 113
PNG 418	CIRAD-CP	RUQ 1309
POUND 7/B (POU)	KEW, UK	RUQ 24
SAN MIGUEL (CHA)	ICG, T	RUQ 142
T85/799	CIRAD-CP	RUQ 855

Inoculations were made using *F. decemcellulare* isolate IMI380504 and *Lasiodiplodia* isolate AC371 on the stem of each plant when the grafted buds were one month old. The inoculation procedure was adapted from Sacristan (1982): there was one inoculation position per plant: near either the top, or middle of the crown. At the inoculation position, a petiole was removed, and then a wound was made with a sterilised scalpel knife to expose part of the wood tissue, before the inoculum was applied. To each wound, a 10-μl spore suspension was applied (macroconidia from two-week old cultures having been previously suspended in sterile distilled water to which a few drops of 0.025 % Tween-20 had been added and adjusted to 1×10^6 spores per ml). Wounds were sealed after inoculation with Parafilm *M* (Pechiney Plastic Packaging, Chicago, IL). The Parafilm was removed 2 weeks after inoculation. Control plants were inoculated with distilled water only. Following inoculation, the plants were placed in a translucent polyethylene enclosure, sprayed with water, and returned to the greenhouse benches, where they were arranged in a randomised complete block design.

Two distinct symptoms of infection were observed in inoculated plants in the current study. These were the distinct leaf and shoot withering, and tissue necrosis within the stem, usually brown in colour with dark or pinkish borders. Formerly, only visual aspects of the internal necrotic lesions were used to describe susceptibility of

germplasm lines (Crowdy, 1947; Owen, 1956) but in the current study various simple, visual, non-destructive assessment methods were also employed. The incubation period, i.e. number of days between inoculation and appearance of symptoms was recorded. Data on the proportion of withered leaves were collected at 4, 8, and 12 weeks after inoculation. Dieback severity on each plant was scored by visual estimation and comparison with the pictorial guide described in Chapter 2. Brown necrotic lesions in stems were measured 12 weeks after inoculation. Due to the destructive nature of the sampling necessary for this assessment, repeated measurements during the growth of the plants were not possible. The percentage area of necrosis on stem sections (cut two millimetres below the point of inoculation) was measured with the aid of a sheet of one-millimetre-scaled tracing graph paper and to assess the internal growth of lesions into the stems, slices were cut out and the brown lesions measured with a hand-held calliper. Sections for light microscopy were cut at 1-2 mm thickness with a kitchen slicer from different distances from the point of inoculation and stained by the technique described by Shipton and Brown (1962): the sections were boiled in alcohol and lactophenol-aniline blue and after one hour of clearing in saturated chloral hydrate and mounting on slides in lactophenol-glycerol, the presence or absence of fungal colonisation was noted. In addition wood fragments were removed sequentially from the point of inoculation and fungal colonisation determined by culturing on PDA. The inoculated stems were removed and destroyed right after the score.

4.3.4. Resistance-indicator data analysis

The essential methods of experimental design and data analysis have been extensively dealt with by Crawley (2005). Data analysis was performed using the four different computer packages: Statistical Package for Social Sciences (SPSS), Minitab, Microsoft Excel and R. Resistance to dieback disease in cocoa was thought to depend on a number of resistance-indicator variables so analysis was made of those considered most likely to influence the overall process, i.e. incubation period, proportion of withered leaves, spread of the fungi within stem tissue, lesion length and width, lesion area on transverse sections and final disease scores 12 weeks after inoculation. Percentage of cross-sectional area of necrosis was transformed by arcsine

square root (Sokal and Rohlf, 1995). The Kruskal-Wallis non-parametric ANOVA was used to assess differences in disease severity scores. Analyses of other variables were done for treatment combinations averaged across replicates: isolate × clone. Box plots (median, 25th and 75th percentiles, and minimum-maximum) and General Linear Model Analysis of Variance (GLM ANOVA) tests were done on measurement data to evaluate the significance of the different variables considered in the study. This was also done to determine the most influential variables: variables were successively tested for significance and only included if the residual sum of squares was significantly reduced. Least significant difference tests were used to analyse the significance of lesion sizes and the changes of withered leaves during the 12 week infection period. No procedure was used to identify and remove outlier data points, and unless otherwise indicated, a significance level of $P \leq 0.05$ was used as arbiter of significant differences between clones. Box-plots analysis was conducted for an overall descriptive presentation of the data. The cocoa genotype responses to either *Fusarium* or *Lasiodiplodia* were compared by employing Pearson's product-moment correlation analysis. Prior to all analyses, the statistics of the absolute severity data were transformed to arcsine square root. The response was considered positive when they showed statistical significance.

4.4. Results

4.4.1. Relative aggressiveness of isolates, lesion development, and variation in susceptibility of clones *in-vitro*

Necrotic lesions, characterised by dark brown colouration developed in all twenty-nine cocoa clones tested. The lesions in stems were darker, and the interface between the lesion and apparently healthy tissue was sharper than in non infected tissue. Analysis of variance detected significant ($P < 0.001$) differential responses of the cocoa genotypes to the three fungal isolates in the resistance tests on stems (Table 17). The LSD values for the isolates (AC845, AC371 and IMI380504) were 0.75, 1.98 and 0.96 mm, respectively. There was also significant genotype × isolate interaction

(Table 17). Sizes of the necrotic lesions caused by the different fungal isolates are summarised in Table 18. Isolate AC845 was more aggressive than the other isolates on only six genotypes, viz., BE 2, GU 125/C, LCTEEN 37/I, PA 107, PA 120 and SC 9 while isolate AC371 caused largest lesions on as many as eleven genotypes (Amelonado, CATIE 1000, CRU 100, CRU 124, GU 136/H, LCTEEN 37/F, MXC 67, NA 149, PA 7, SC 20 and UF 677). Isolate IMI380504 ranked highest on nine clones- AMAZ 15/15, AMAZ 3/2, AMAZ 5/2, EET 59, GU 123/V, ICS 43, PA 137, POUND 7/B and T85/799.

Table 17. Results (F and P-values) from univariate ANOVA on lesion size in cocoa stem segments from laboratory experiments. Square-root transformed data were used for analysis.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Cocoa genotype	28	2132.72	76.17	15.6752	< 2.2 e-16 ***
Fungal isolate	2	242.11	121.05	24.9125	1.254 e- 10 ***
Genotype : Isolate	56	2117.22	37.81	7.7806	< 2.2 e-16 ***
Residuals	261	1268.25	4.86		

Inoculation with AC371 caused the largest stem lesions *in-vitro* (mean = 4.33 mm, SE = 0.97, min = 0.25 mm, max = 20.7 mm) while isolate AC845 caused the smallest (mean = 2.41 mm). Considerably larger lesions were observed in genotypes UF 677 and LCTEEN 37/F than in many of the other clones 14 days after inoculation. The lesion lengths for clone LCTEEN 37/F were 2.2, 18.5 and 11.7 mm against isolates AC845, AC371 and IMI380504, respectively. The lesion lengths for clone SCA 6 against the three isolates were respectively 0.5, 0.5 and 0.2 mm. Eight genotypes were placed in one group and they represent the lowest level of resistance in this trial (mean lesion across isolates > 4 mm). Eleven genotypes (e.g. AMAZ 3/2, AMAZ 5/2, BE 2, CATIE 1000, CRU 124, GU 125/C, ICS 43, MXC 67, SC 9, SCA 6 and T85/799) that showed the best performance with lesion sizes < 2mm were placed in another group while the intermediate group, included ten genotypes (Table 18). The extent to which the performance of the three fungal isolates was correlated is shown in Figure 45 to Figure 47. All were positive, albeit weakly so.

Table 18. Variation in the severity of necrosis (visible lesion length, mm) caused by two *Lasiodiplodia isolates and a standard *F. decemcellulare*[+] isolate on inoculated cocoa genotypes *in-vitro* 14 days after inoculation.**

Cocoa genotype	Visible lesion length, isolate means[1]			Mean lesion size ± se	Rank
	AC845*	AC371*	IMI 380504[+]		
AMAZ 15/15	2.00	2.00	2.75	2.25 ± 0.25[b]	14th
AMAZ 3/2	0.75	1.00	1.25	1.00 ± 0.14[c]	5th
AMAZ 5/2	0.50	1.00	1.50	1.16 ± 0.09[c]	6th
AMELONADO	4.75	8.75	4.75	6.08 ± 0.76[a]	26th
BE 2	1.75	1.00	0.75	1.16 ± 0.30[c]	7th
CATIE 1000	2.25	3.50	0.25	2.00 ± 0.94[c]	11th
CRU 100	4.00	8.00	5.00	5.66± 1.20[a]	24th
CRU 124	0.25	2.25	1.25	1.25 ± 0.57[c]	8th
EET 272	2.75	5.75	5.75	4.75 ± 1.00[a]	23rd
EET 59	0.50	0.25	5.75	2.16 ± 1.79[b]	13th
GU 123/V	4.00	0.75	6.25	3.66 ± 1.59[b]	20th
GU 125/C	2.25	0.75	1.25	1.41 ± 0.44[c]	9th
GU 136/H	4.00	6.25	2.75	4.33 ± 1.02[a]	22nd
ICS 43	0.25	0.50	1.00	0.58 ± 0.22[c]	3rd
LCTEEN 37/F	2.25	18.5	11.75	10.8 ± 4.71[a]	29th
LCTEEN 37/G	2.25	5.50	1.00	2.91 ± 1.34[b]	18th
LCTEEN 37/I	4.00	2.00	1.50	2.50 ± 0.76[b]	15th
MXC 67	0.75	3.75	1.50	2.00 ± 0.90[c]	10th
NA 149	8.25	9.50	0.50	6.08 ± 2.81[a]	27th
PA 107	5.25	1.50	1.25	2.66 ± 1.29[b]	17th
PA 120	5.50	1.00	4.74	3.74 ± 1.39[b]	21st
PA 137	1.75	1.75	2.75	2.08 ± 0.33[b]	12th
PA 7	2.00	4.00	3.00	3.00 ± 0.57[b]	19th
POUND 7/B	0.25	3.25	4.25	2.58 ± 1.20[b]	16th
SC 20	5.25	11.25	0.50	5.66 ± 3.11[a]	25th
SC 9	0.75	0.50	0.50	0.58 ± 0.08[c]	2nd
SCA 6	0.50	0.50	0.25	0.41 ± 0.08[c]	1st
T85/799	0.50	0.25	1.50	0.75 ± 0.38[c]	4th
UF 677	0.75	20.7	4.75	8.73 ± 6.09[a]	28th
Mean	2.41	4.33	2.75		
LSD α =0.05	0.75	1.98	0.96		

[1]Data points are means calculated on four replicates.
[a]Susceptible group.
[b]Intermediate grou.p
[c]Tolerant group.
Mean lesion on water treated control was 0.4 mm

4.4.2. Fungal colonisation of detached stem segments

Colonisation occurred in all 29 cocoa genotypes screened and there were differences between the fungal isolates (Table 19). Mean colonisation distance from the source was 1.8, 9.6 and 6.0 mm for isolates AC845, AC371, and IMI380504, respectively. On genotypes PA 137, PA 7, SC 20, SCA 6 and T85/799, isolate AC845 colonised the least distance (0.3 mm) compared with the other isolates. Isolate AC845 progressed only slightly further on BE 2, EET 272, EET 59, GU 136/H, LCTEEN 37/I and SC 9 (0.5 mm) and AMAZ 3/2, AMAZ 5/2, CRU 100, and PA 120 (1.0

mm). Growth of isolate AC371 was severely restricted on AMAZ 3/2, PA 7 and SCA 6 (0.5 mm) but it developed extensively on UF 677, POUND 7/B and GU 123/V among others. Least fungal colonisation across isolates was found in genotypes SCA 6 (0.4 mm), EET 272 (0.6 mm) and MXC 67 (0.6 mm) whereas the most colonised genotypes were NA 149 (11.6 mm), UF 677 (12.9 mm) and GU 123/V (16.1 mm). Genotypes CRU 124, EET 272, ICS 43, SC 20 and SCA 6 were similar with respect to their response to isolate IMI380504 (0.5 mm). Isolates within the same genus, for example AC845 and AC371 differed in amount of colonisation except on genotype MXC 67, for which no significant difference between the two isolates was detected. In SCA 6, isolate AC845 was detected only in the 0.3-mm-long section next to the inoculation point while isolates AC371 and IMI380504 were both detected 0.5 mm away. On clone GU 123/V, the respective distances of isolation of the three fungi were 16.8, 18.8 and 12.8 mm. A general observation of the cultures indicated a gradient in the amount of fungal biomass (mycelial growth), with the highest levels immediately adjacent to the inoculation point. Figure 48 to Figure 50 and appendix 1 show correlation between colonisation of the three isolates on the cocoa genotypes.

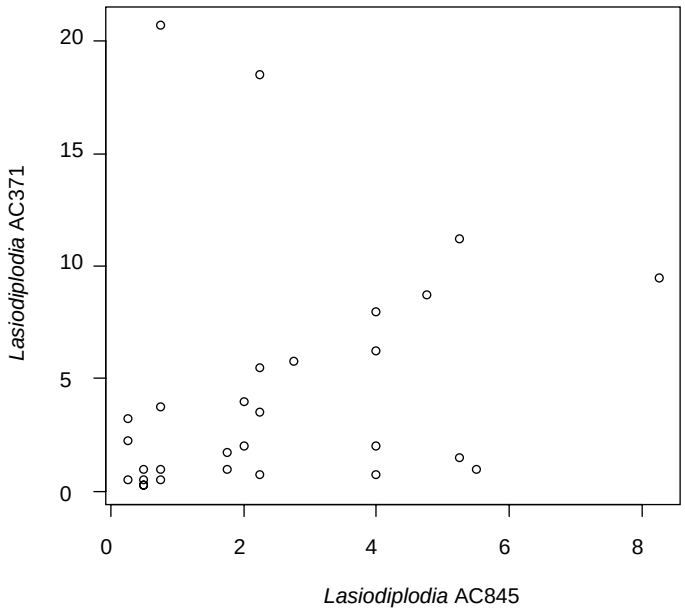

Figure 45. Pearson's product-moment correlation of necrotic lesions by *Lasiodiplodia theobromae* AC845 and *Lasiodiplodia* AC371 on different cocoa germplasm lines. r = 0.3, t = 1.3534, df = 27, p-value = 0.1872; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from -0.1261103 to 0.5662434.

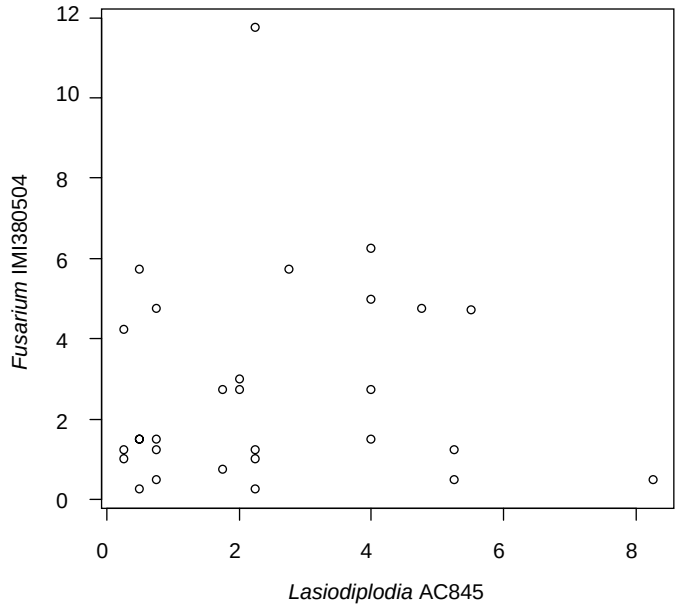

Figure 46. Pearson's product-moment correlation of necrotic lesions by *Lasiodiplodia* AC845 and *Fusarium decemcellulare* IMI380504 on different cocoa germplasm lines. r = 0.1, t = 0.3023, df = 27, p-value = 0.7648; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from -0.3151421 to 0.4157285.

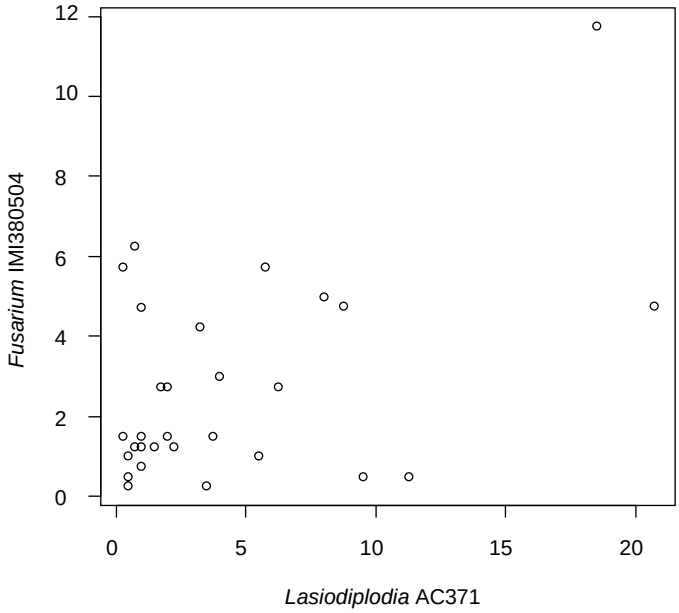

Figure 47. Pearson's product-moment correlation of necrotic lesions by *Lasiodiplodia* AC371 and *Fusarium decemcellulare* IMI380504 on different cocoa germplasm lines. r = 0.5, t = 2.9235, df = 27, p-value = 0.006925; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from 0.1509704 to 0.7263203.

Table 19. Variation in the distance of spread of viable mycelia (colonisation, mm) of *Lasiodiplodia* and *F. decemcellulare* within cocoa genotypes *in-vitro* 14 days after inoculation.

Genotype	Distance from inoculation point to edge of colonisation[1]			Mean distance of colonisation ± se	Rank
	AC845*	AC371*	IMI 380504		
AMAZ 15/15	7.25	9.00	10.00	8.75 ± 0.80	24th
AMAZ 3/2	1.00	0.50	1.00	0.83 ± 0.16	5th
AMAZ 5/2	1.00	5.00	8.75	4.91 ± 2.23	12th
AMELONADO	2.75	13.75	8.25	8.25 ± 3.17	22nd
BE 2	0.50	9.75	13.75	8.00 ± 3.92	21st
CATIE 1000	1.25	10.25	8.50	6.66 ± 2.75	16th
CRU 100	1.00	17.50	4.50	7.66 ± 5.01	19th
CRU 124	2.50	17.00	0.50	6.66 ± 5.19	17th
EET 272	0.50	0.75	0.50	0.58 ± 0.08	2nd
EET 59	0.50	16.00	6.75	7.75 ± 4.50	20th
GU 123/V	16.75	18.75	12.75	16.08 ± 1.76	29th
GU 125/C	0.25	14.25	1.00	5.16 ± 4.54	15th
GU 136/H	0.50	4.75	4.50	3.25 ± 1.37	10th
ICS 43	0.75	1.25	0.50	0.83 ± 0.22	6th
LCTEEN 37/F	7.25	17.75	2.50	9.16 ± 4.50	25th
LCTEEN 37/G	0.75	17.50	15.50	11.25 ± 5.28	26th
LCTEEN 37/I	0.50	2.00	9.25	3.91 ± 2.70	11th
MXC 67	0.75	0.75	0.25	0.58 ± 0.16	3rd
NA 149	2.00	18.50	14.25	11.58 ± 4.94	27th
PA 107	0.25	1.50	1.25	1.00 ± 0.38	7th
PA 120	1.00	8.00	7.00	5.33 ± 2.18	13th
PA 137	0.25	9.00	11.75	7.00 ± 3.46	18th
PA 7	0.25	0.50	1.25	0.66 ± 0.30	4th
POUND 7/B	0.75	18.50	5.75	8.33 ± 5.28	23rd
SC 20	0.25	19.00	0.50	6.58 ± 6.20	14th
SC 9	0.50	2.75	2.00	1.75 ± 0.66	8th
SCA 6	0.25	0.50	0.50	0.41 ± 0.08	1st
T85/799	0.25	3.00	3.00	2.08 ± 0.91	9th
UF 677	1.25	19.75	18.00	12.98 ± 5.90	28th
Mean	1.81	9.56	6.00		
LSD α =0.05	1.26	2.76	2.02		

[1]Data points are means calculated on four replicates.

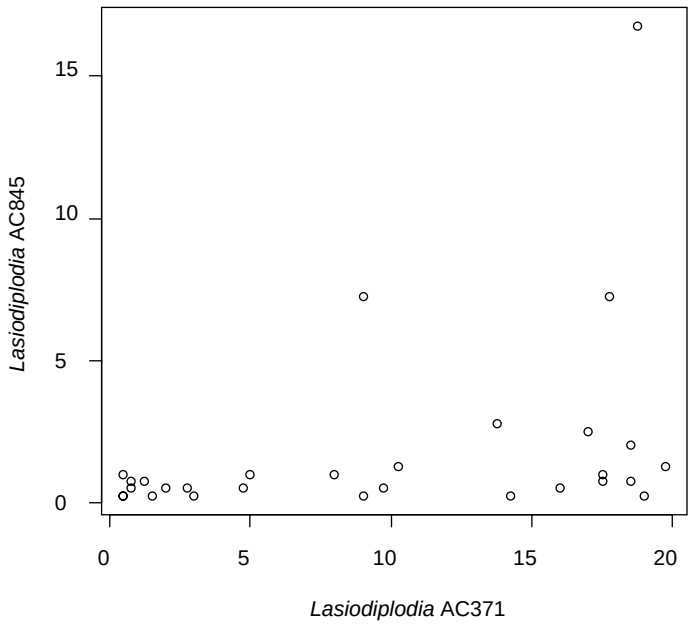

Figure 48. Pearson's product-moment correlation of stem colonisation by *L. theobromae* AC845 and *L. pseudotheobromae* AC371 on different cocoa germplasm lines. r = 0.4, t = 1.9941, df = 27, p-value = 0.05633; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from -0.009459452 to 0.640665281.

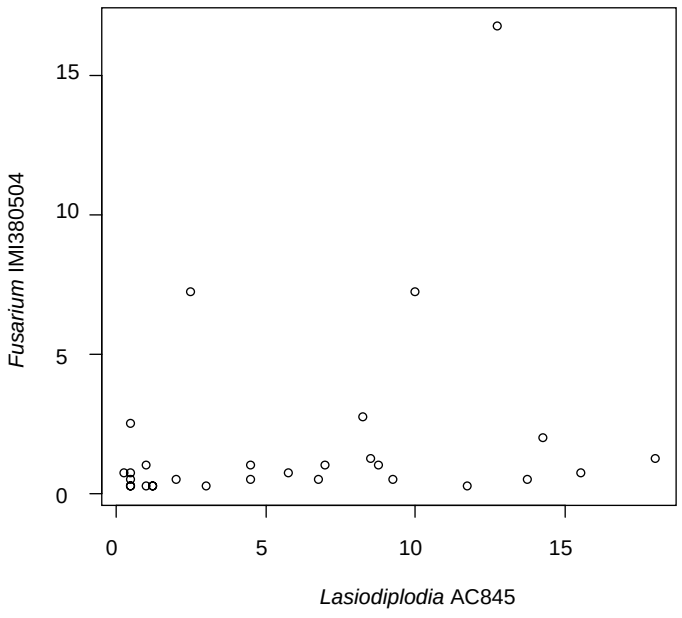

Figure 49. Pearson's product-moment correlation of stem colonisation by *Lasiodiplodia theobromae* AC845 and *F. decemcellulare* IMI380504 on different cocoa germplasm lines. r = 0.3, t = 1.4232, df = 27, p-value = 0.1661; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from -0.1133151 to 0.5749977.

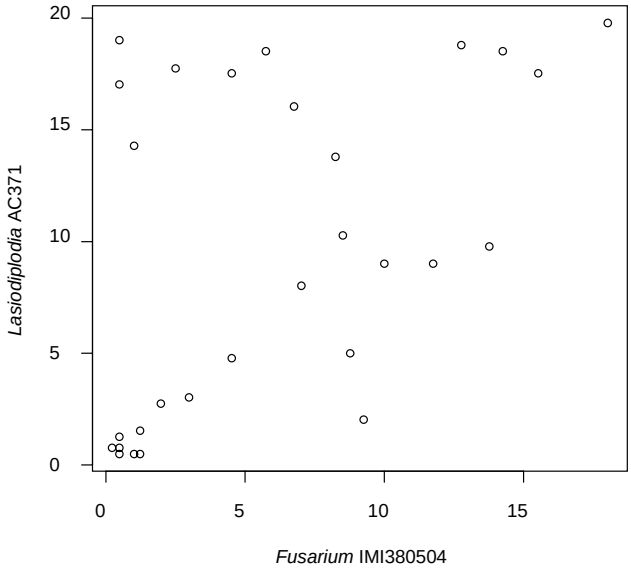

Figure 50. Pearson's product-moment correlation of stem colonisation by *Lasiodiplodia pseudotheobromae* AC371 and *F. decemcellulare* IMI380504 on different cocoa germplasm lines. r = 0.5, t = 2.8077, df = 27, p-value = 0.009154; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from 0.1318525 to 0.7169720.

4.4.3. Comparison of lesion length and fungal colonisation in inoculated stem segments *in-vitro*

Pearson's correlation coefficients between lesion length and distance of colonisation for the different isolates were positive and significant (Figure 51 to Figure 53). This suggests that a general increase or decrease of lesion length in stems was associated with either an increase or a decrease of colonisation. The observation from this trial has to be interpreted cautiously because while the results of some clones were consistent across the three fungal isolates (for both necrotic lesion and colonisation assessment methods), there was no definite trend on other genotypes. For example, SCA 6 ranked first (most resistant) in both the lesion size and tissue colonisation methods. AMAZ 3/2 also consistently expressed partial resistance in both methods, ranking the fifth most resistant. Other genotypes such as UF 677 and Amelonado were consistently ranked susceptible in both evaluations. On the contrary, the genotype EET 272, showed good resistance in the tissue colonisation method, but high

susceptibility in the lesion size method. Among the evaluated clones, the study has identified a few that developed only small necrotic lesions; SCA 6, SC9 and ICS 43 seemed to be the most promising ones as judged by the detached stem evaluation method. Some clones did not display strong visual symptoms but the distances of colonization were relatively high (e.g. IMI380504 on NA 149 and UF 677), which suggests latent and symptomless growth of fungi in host tissues. In other combinations, however, (e.g. isolate AC371 on POUND 7/B and isolate IMI380504 on LCTEEN 37/F), the reverse is the case.

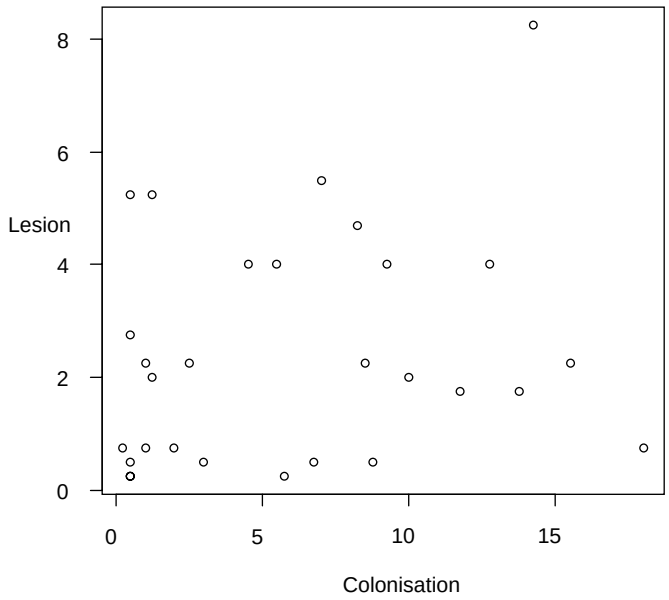

Figure 51. Pearson's product-moment correlation between lesion length and distance of colonisation by isolate AC845. r = 0.2, t = 1.1726, df = 27, p-value = 0.2512; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from -0.1592180 to 0.5428425.

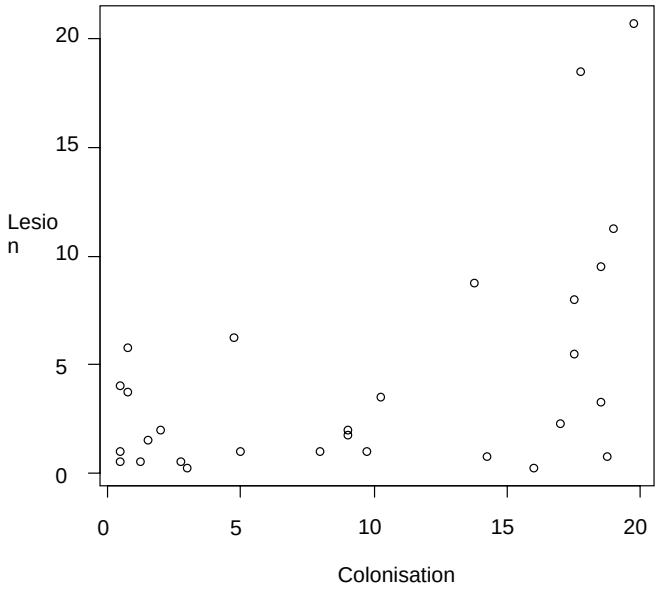

Figure 52. Pearson's product-moment correlation between lesion length and distance of colonisation by isolate AC371. r = 0.5, t = 3.1084, df = 27, p-value = 0.004397; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from 0.1809016 to 0.7405407.

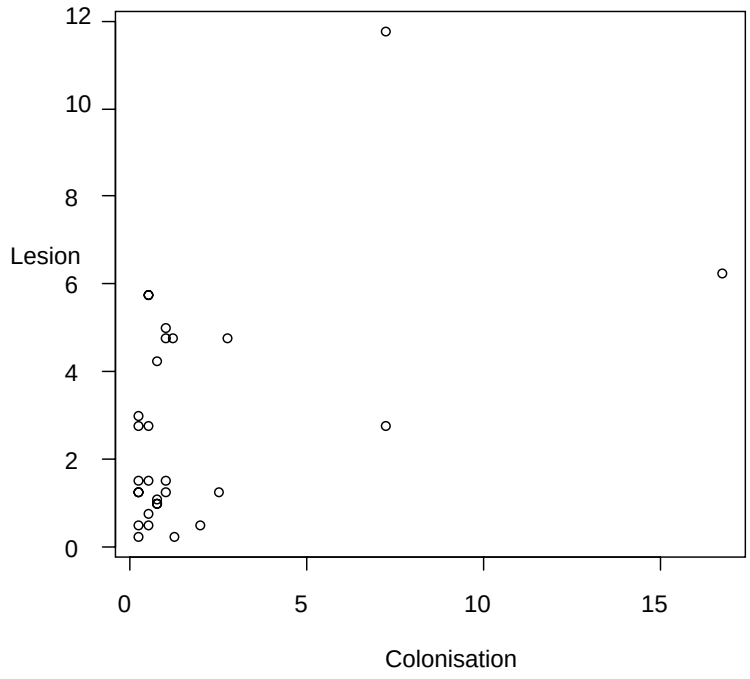

Figure 53. Pearson's product-moment correlation between lesion length and distance of colonisation by isolate IMI380504. r = 0.4, t = 2.8741, df = 27, p-value = 0.007804; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from 0.1428571 to 0.7223788.

4.4.4. Greenhouse evaluations

Fourteen grafted cocoa genotypes were selected for testing in the greenhouse to compare their susceptibility to *F. decemcellulare* isolate IMI 380505 and *Lasiodiplodia* isolate AC371 infection. Amelonado with high susceptibility (Chapter 2) was included for comparison. The results are given in Table 20 and Table 21. Isolates of both *F. decemcellulare* and *Lasiodiplodia* were able to colonise and develop on some clones, whereas on other clones, pathogen colonisation seems to have been hindered thereby resulting in smaller, necrotic lesions. However, the point of inoculation of all clones became swollen and a large amount of gum was produced, presumably as part of the plant defence response to combat further fungal colonisation. Analysis showed non-significant differences in the levels of infection between the two fungi as lesion measurements and dieback scores were comparable. Both means and variances (indicated by LSD) are similar in the two tables.

Table 20. Components of resistance in fifteen cocoa germplasm clones varying in susceptibility to *Fusarium decemcellulare* in greenhouse evaluations.

Description of clones	Components of resistance								
	Incubation period (days)[a]	Dieback Score (mean)[b]	Percentage of withered leaves			Lesion width (mm)	Lesion length (mm)	Distance of fungal spread (mm)	% stem cross sectional area with necrosis [c]
			4 wks	8 wks	12 wks				
AMELONADO[d]	9	8.4	51.6	61.5	88.4	1.90	35.12	26.1	36.0
CATIE 1000	30	1.8	3.7	4.0	4.3	0.36	16.24	13.2	6.2
EET 59	12	6.0	32.2	41.6	52.8	1.78	33.00	29.4	31.0
ICS 1	23	4.6	25.3	30.5	37.9	1.56	27.16	25.1	26.0
ICS 95	22	5.0	25.7	33.6	40.5	0.96	25.80	20.7	23.0
IMC 67	27	3.0	16.8	20.0	21.6	1.24	17.68	14.5	23.0
LCTEEN 37/A	11	7.2	50.0	58.2	67.6	1.32	32.80	27.2	28.0
LCTEEN 37/F	12	8.4	54.6	67.6	90.8	1.78	34.32	32.0	31.7
LCTEEN 37/G	9	7.8	60.9	72.5	88.7	1.15	35.74	29.3	28.2
MXC 67	25	2.6	8.5	12.3	10.8	1.14	20.62	16.0	16.3
PA 7 (PER)	22	6.2	21.4	43.1	53.3	1.06	24.70	22.8	19.5
PNG 418	17	6.0	25.5	45.3	53.9	1.90	20.82	16.3	23.8
POUND 7/B	18	4.4	24.5	31.0	35.1	1.22	28.20	22.1	19.9
SAN MIGUEL	21	3.8	14.0	16.0	21.6	1.30	21.64	20.8	23.4
T85/799	25	2.4	6.6	10.8	9.5	1.56	16.72	11.1	16.5
Mean	19	5.1	28.1	36.5	45.1	1.34	26.03	21.7	23.5
LSD α =0.05	4	1.2	10.1	12.0	16.1	0.22	3.84	3.5	4.1

[a] Days after inoculation to first visual symptoms; means rounded to the nearest one day.
[b] Mean dieback scores recorded using a nine-point disease scale (1 = no visible symptoms, 9 = dead plant).
[c] Real figures; analysis was done after arc sine transformation.
[d] Susceptible variety.

Table 21. Components of resistance in fifteen cocoa germplasm clones varying in susceptibility to *Lasiodiplodia* in greenhouse evaluations.

Description of clones	Components of resistance								
	Incubation period (days)[a]	Dieback Score (mean)[b]	Percentage of withered leaves [c]			Lesion width (mm)	Lesion length (mm)	Distance of fungal spread (mm)	% stem cross sectional area with necrosis [c]
			4 wks	8 wks	12 wks				
AMELONADO[d]	9	9.0	38.6	60.2	100.0	2.18	39.16	26.0	30.6
CATIE 1000	31	2.4	5.1	6.5	10.8	0.70	16.88	14.3	6.1
EET 59	11	6.0	40.0	48.8	60.0	2.22	34.28	29.9	35.6
ICS 1	23	4.0	17.4	22.7	32.1	1.18	26.52	22.3	22.3
ICS 95	18	4.4	25.4	29.9	38.4	1.28	26.44	20.6	24.3
IMC 67	28	2.4	9.4	11.3	13.1	1.20	20.22	15.8	23.3
LCTEEN 37/A	8	8.4	58.8	67.9	89.7	1.24	36.30	31.0	24.4
LCTEEN 37/F	10	9.0	68.7	74.3	100.0	1.56	36.36	34.0	34.0
LCTEEN 37/G	12	8.4	57.5	66.3	80.6	1.00	31.14	28.0	26.1
MXC 67	21	2.2	5.7	6.4	9.8	1.18	22.56	20.2	19.4
PA 7 (PER)	19	6.6	41.4	51.5	63.9	1.08	25.46	23.3	22.7
PNG 418	21	4.4	15.0	19.3	25.6	2.00	21.34	18.4	31.8
POUND 7/B	13	6.0	29.6	41.8	50.3	1.94	26.80	23.6	28.6
SAN MIGUEL	22	4.4	21.5	24.0	31.1	1.80	21.64	17.7	33.2
T85/799	21	2.4	5.9	9.0	9.9	1.10	17.44	12.8	16.1
Mean	18	5.3	29.3	35.9	47.6	1.44	26.83	22.5	25.2
LSD α =0.05	4	1.3	11.5	13.3	18.2	0.25	3.95	3.4	4.2

[a] Days after inoculation to first visual symptoms; means rounded to the nearest one day.
[b] Mean dieback scores recorded using a nine-point disease scale (1 = no visible symptoms, 9 = dead plant).
[c] Real figures; analysis was done after arc sine transformation.
[d] Susceptible variety.

Figure 54 shows an example of a budded clone used for the greenhouse resistance screening. The rootstock is a parental Amelonado plant while the attached scion, marked by an arrow, is from LCTEEN 37/F. The scion is two month old and shows a strong bud-rootstock union. Similar results were obtained with the other clones, but there were instances when the implant was unsuccessful and budding had to be repeated.

Figure 54. An eight-week-old grafted clone attached to an Amelonado rootstock.

In the greenhouse study, clones were evaluated for incubation period, lesion size and dieback, and the measures of shorter incubation periods, larger lesions, and high dieback scores implied susceptibility. Visual ratings for dieback of the shoot generally corresponded to ratings previously obtained from greenhouse studies on susceptible Amelonado cocoa. Disease was not apparent during the first week after inoculation, with nearly all the plants able to produce new flush leaves. However, from the second week, characteristic foliar symptoms of the disease were observed. Disease symptoms were characterised by wilting of apical leaves and necrosis, which developed within the stem from the point of inoculation downwards. The incubation period ranged from

eight to 30 days during which time drooping and wilting of the leaves became visible. After the initial disease symptoms, the most susceptible plants were dead after 12 weeks. Disease scores varied between cocoa clones and ranged from 1.8 to 9.0 (Table 20 and Table 21). On CATIE 1000 and T85/799, only 40 percent of the plants developed foliar symptoms with an average disease index of 1.8 to 2.4 while the remaining plants had no or barely detectable expression of dieback. The five populations from LCTEEN 37/A, LCTEEN 37G, LCTEEN 37/F, SANMIGUEL and POUND 7/B developed the most severe symptoms which were similar to those shown by the infected Amelonado control (Table 20 and Table 21). Between these extremes, the populations from PA 7 and IMC 67 had intermediate levels of symptoms. When plant sections from cocoa germplasm with symptoms were tested for the presence of the fungi, colonies were detected although the density of viable hyphae decreased away from the point of inoculation. This observation was true even in symptomless plants. Despite this, *F. decemcellulare* or *Lasiodiplodia* was still recovered from stem tissues 12 weeks after inoculation. These fungi were likely to have come only from within the necrotic region around the point of inoculation, where the fungi were confined by the host defence response. Neither the xylem in necrotic areas of stems nor any other tissues were seen to be filled with fungal hyphae, but rather they appeared to be filled with gummy substances. This material probably contained phenolic compounds that were produced as a host defence response.

The assumption for a parametric ANOVA test was however not supported on these disease scores. Therefore, a Kruskal-Wallis non-parametric test was carried out on the data. The output (Table 22) confirms the test used. The summary information of each cocoa clone is given, N being the number of observations as well as the median and mean rank. The Z-value is used in the test calculation. The last two lines give the tests results depending on how tied observations in the data were treated. Here, the test statistic obtained was greater than the critical value H (48.29, d.f. = 13, $P < 0.001$) and therefore the null hypothesis was rejected. The analysis suggests that at least one of the clones yielded a median score value was different from that yielded by at least one of the other clones. The significant result ($P < 0.001$) from the Kruskal-Wallis test is shown in Table 22.

A microscopic observation and measurements was performed on stem sections to determine the distribution of necrotic tissues. In transverse section, under light microscopy, the stem showed a simple structure. Staining of a transverse section of the stem with aniline blue lactophenol revealed the presence of necrotic areas, mainly inward from the cortical layer and the xylem vessels in the vascular bundle towards the pith.

4.4.4.1 Incubation period

The clones used in the study expressed reasonable consistency in the various resistance components measured. Incubation period decreased distinctly from resistant to susceptible clones. The apparently resistant clone CATIE 1000 had the longest incubation period of 30 days compared to 8 days for LCTEEN 37/A.

4.4.4.2. Dieback assessment

The dieback scores for the two lots of genotypes, one receiving isolate AC371, the other isolate IMI380504 were compared. The individual scores are recorded in (Table 23). The calculations for the test of significance are given at the foot of the table. With 28 degrees of freedom, the value of *t* is not significant at the 1 % level. Isolate AC371 gave comparable average dieback scores as IMI380504 and therefore, the dieback susceptibility of the 15 cocoa genotypes tested was not significantly different between the two isolates used in the study ($P \leq 0.05$). The maximum disease was recorded on Amelonado, LCTEEN 37/F and LCTEEN 37/G with mean score of 9.0 followed by EET 59, PA 7, and PNG 418 with disease scores ranging between 5 and 7. Four clones CATIE 1000, IMC 67, MXC 67, and T85/799 had scores below 3.0. Two clones, Amelonado, and LCTEEN 37/F exhibited maximum susceptibility against *L. theobromae*, while minimum disease incidence was observed in CATIE 1000 against *F. decemcellulare.* An example of dieback symptom is shown in Figure 55.

Table 22. Kruskal-Wallis test of data from greenhouse experiment to evaluate dieback severity scores in different cocoa genotypes.

Clones	N	Median	Ave Rank	Z*
Amelonado	10	9.000	119.5	3.32
CATIE 1000	10	1.000	20.0	-4.18
EET 59	10	6.000	92.3	1.27
ICS 1	10	6.000	66.4	-0.69
ICS 95	10	6.000	70.5	-0.37
IMC 67	10	4.500	58.3	-1.30
LCTEEN 37/A	10	6.000	84.2	0.66
LCTEEN 37/F	10	9.000	119.5	3.32
LCTEEN 37/G	10	6.000	92.3	1.27
MXC 67	10	2.000	56.9	-1.41
PA 7	10	3.000	51.5	-1.81
PNG 418	10	7.500	95.7	1.52
POUND 7/B	10	6.000	82.8	0.55
SAN MIUEL	10	6.000	90.3	1.12
T85/799	10	1.000	32.3	-3.25
Overall	150		75.5	

H = 60.79 DF = 14 P = 0.000
H = 66.36 DF = 14 P = 0.000 (adjusted for ties)
*The Z value for each group is the standardized value of the deviation between the $\check{R}i$ for the ith group and its expected value $(N + 1)/2$ under the null hypothesis.

Table 23. Testing the difference between the means of dieback scores caused by isolate AC371 and IMI380504.

Cocoa genotype	Dieback scores	
	AC371	IMI380504
Amelonado	9.0	8.4
CATIE 1000	2.4	1.8
EET 59	6.0	6.0
ICS 1	4.0	4.6
ICS 95	4.4	5.0
IMC 67	2.4	3.0
LCTEEN 37/A	8.4	7.2
LCTEEN 37/F	9.0	8.4
LCTEEN 37/I	8.4	7.8
MXC 67	2.2	2.6
PA7 (PER)	6.6	6.2
PNG 418	4.4	6.0
POUND 7/B	6.0	4.4
SANMIGUEL	4.4	3.8
T85/799	2.4	2.4
ΣX	80.0	77.6
n	15	15
Mean X'	5.33	5.17
ΣX^2	514.88	468.96
$(\Sigma X)^2/n$	426.66	401.45
Σx^2	88.22	67.51
df	14	14

Pooled $s^2 = \frac{88.22 + 67.51}{14 + 14} = 5.561$ df = 28

$sx_1'\text{-}x_2' = \sqrt{2s^2/n} = \sqrt{2}(5.561)/15 = 0.86$

$t = (X_1'\text{-}X_2')/\ sx_1'\text{-}x_2' = 0.16/0.86 = 0.186$

95 % confidence limits for $\mu_1\text{-}\mu_2$ were
$0.16 \pm t_{0.05}\, sx_1'\text{-}x_2'$

0.16 – (2.048)(0.86) = -1.6 to 1.92

4.4.4.3. Distance of fungal spread (colonisation)

In both the *Fusarium decemcellulare* (IMI380504) and *Lasiodiplodia* (isolate AC371) inoculations the results showed evidence of variation in fungal colonisation of stem tissue between the different cocoa genotypes. Analysis of variance yielded highly significant ($P < 0.05$) clonal effects for Isolates IMI380504 and AC371 (Table 20 and Table 21). Therefore, for both pathogens, the distance of fungal stem colonisation measured depended on the cocoa genotype. LCTEEN 37/F was highly colonised by both pathogens while T85/799 and CATIE 1000 were the least colonised. There was a

general trend for shorter distance of hyphal growth than visible lesions. That is, viable hyphae were only recovered from within the visibly diseased wood.

Figure 55. An example of the type of dieback symptoms caused by *L. Pseudotheobromae* in young plants of clones PNG 418 and LCTEEN 37/G infected 12 weeks after inoculation in the greenhouse.

4.4.4.4. Assessment of necrotic lesions

Following the use of visible symptoms for scoring of dieback severity, internal tissues of stems (Figure 57) were then inspected for damage at the end of the experiment, with the view to correlating dieback damage with internal tissue collapse. Table 20 and Table 21 show the reactions induced by the two fungal isolates on the 15 cocoa germplasm types. All 14 clones were susceptible to the two fungi and showed some level of necrotic lesions. The lesions were irregularly elongated often with a pinkish-brown colouration. Inoculation with isolate AC371 appeared to have caused a greater across clone mean lesion length than isolate IMI380504, but the differences were not significant ($P = 0.159$). Generally, the across-isolate lesion dimensions were smaller on CATIE 1000 and T85/799 than on the other clones. Significant clone × isolate interactions were not detected ($P > 0.05$) for lesion length, lesion width nor for the

cross-sectional area of stem surface showing necrosis. This may be partly because a similar response to infection in the different germplasm lines was seen when the plants were inoculated with either pathogen as mean lesion dimensions on identical clones were similar against both isolates. Overall, there was significant correlation between results from the two isolates tested (Figure 60–Figure 62). The ANOVA showed that there were significant differences ($P < 0.05$) between the cocoa clones used. Amelonado, EET 59, LCTEEN 37/A, LCTEEN 37/F, LCTEEN 37/G and SAN MIGUEL were the most severely damaged while CATIE 1000, T85/799, and MXC 67 were the most resistant against the two isolates with respect to necrotic lesion development.

Nine cocoa genotypes (Amelonado, CATIE 1000, EET 59, LCTEEN 37/F, LCTEEN 37/G, MXC 67, PA 7, POUND 7/B and T85/799) were tested in the laboratory as well as the greenhouse. The correlation coefficient between the laboratory assay and the greenhouse test of necrotic lesions was 0.56 (n=9) but this was not significant (Figure 56).

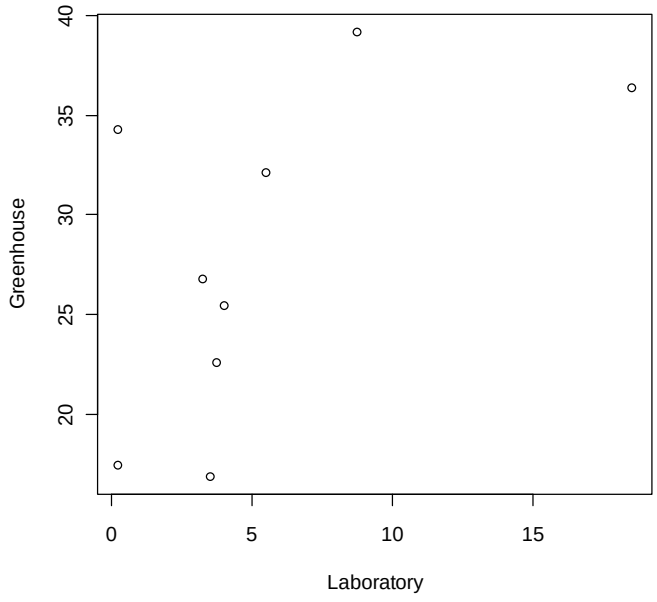

Figure 56. Pearson's product-moment correlation of necrotic lesions caused by isolate AC371 on corresponding cocoa germplasm lines. r = 0.5, t = 1.833, df = 7, p-value = 0.1095; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from -0.1521858 to 0.8950835.

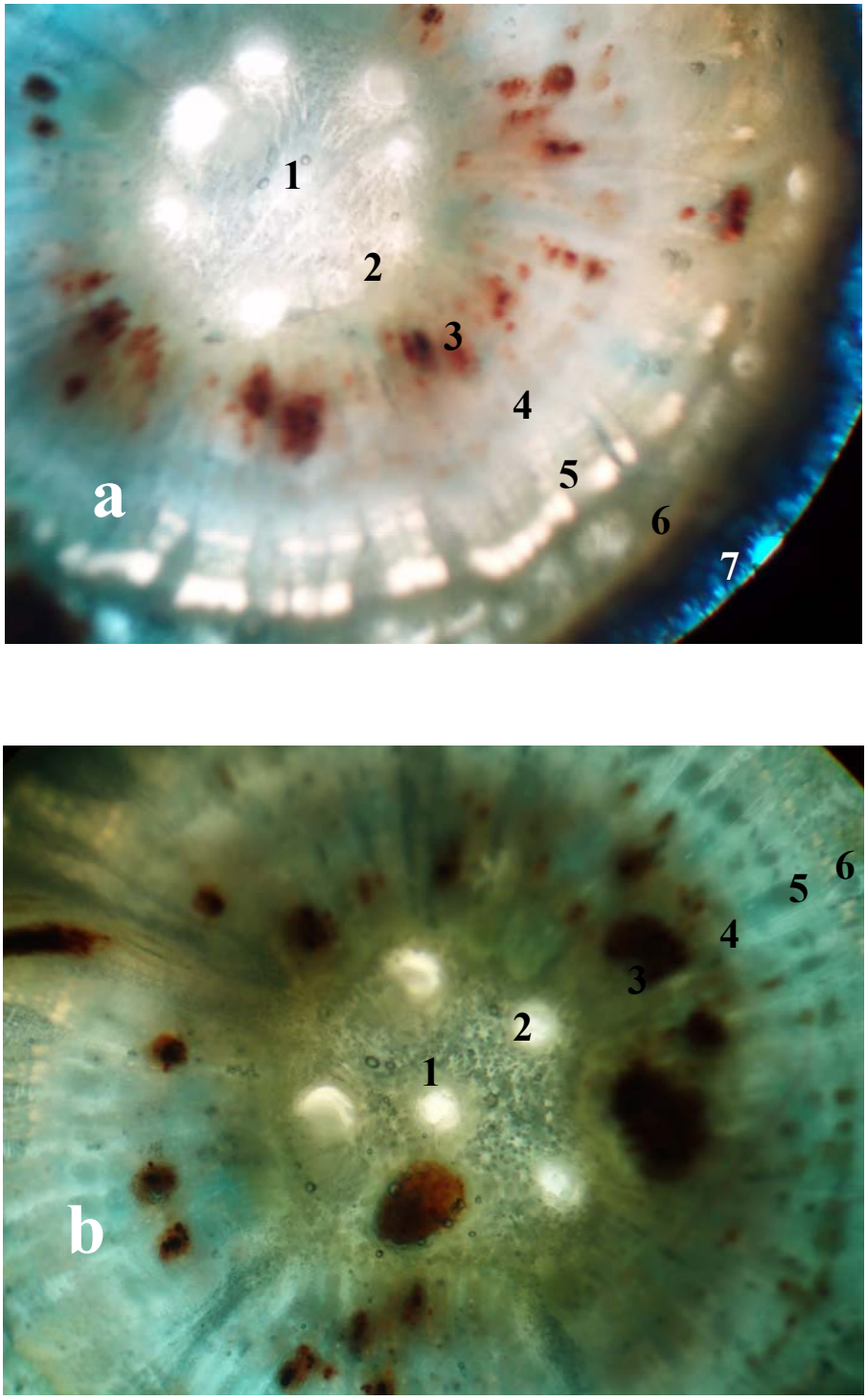

Figure 57. Cross sections of stems of 6-month-old budded cocoa showing necrosis of the xylem tissue; a. clone T85/799 12 weeks after inoculation with isolate AC371 (*L. pseudotheobromae*); b. clone LCTEEN 37/F 12 weeks after inoculation with isolate AC371. 1 = pith, 2 = protoxylem, 3 = xylem, 4 = phloem, 5 = schlerenchyma (blast fibre) - supporting tissue, 6 = cortex, 7 = epidermis. Dark brown portions are necrotic lesions as a consequence of infection by *Fusarium* and *Lasiodiplodia* species.

Box and whisker plots (Figure 58) constructed to examine the differences between the clones across the eight measureable resistance variables confirmed the above observation. Figure 58 shows the median, 25^{th}, 75^{th} percentiles and maximum and minimum values for four variables on four clones inoculated with isolate AC371. From the box-plots, incubation period, lesion length and width and the cross sectional area of stem with necrosis showed significant differences between clones. The analysis of variance (ANOVA) indicated significant differences among the cocoa clones for each resistance component (Table 20 and Table 21).

Further insight could be gained using the correlation matrix in Figure 59, where the rows have the 'response' variable (the *y* axis) as labelled by the variable name that appears in that row and the columns, the 'explanatory' variables as labelled by the variable name that appears in that column. There was a strong correlation between most of the resistance components evaluated (Figure 59) as well as between the results of the two fungal isolates (Figure 60 to Figure 62). There was also a close correlation of dieback scores against some of the other resistance variables (Figure 63 to Figure 66) which is biologically plausible, because shorter incubation period and larger lesion size both reflect the activity of the pathogen. The inward growth of lesions (estimated from percentage area of systemic lesion) was positively correlated with the other resistance components, which were positively correlated with one another but negatively correlated with incubation period. However, the relationship between lesion width and the proportion of withered leaves after four, eight, and twelve weeks is not clear. The data showed that there was a rapid increase in the percentage of withered leaves to around 25 % at four weeks followed by a slower increase such that by the twelfth week weaker clones had reached a value close to 100 percent. Although strongly positively correlated with each other (Figure 59) the withered leaf values at 4, 8 and 12 weeks often seemed independent of the other parameters measured, with marked scatter of points tending to obscure any underlying correlation. Within the approximations made, the mean dieback score for the less susceptible clones was less than 3.0 for the two fungal isolates. Amelonado was the only clone rated most susceptible against isolate IMI380504 whereas two other clones, LCTEEN 37/F and LCTEEN 37/G were similar to Amelonado when inoculated with isolate AC371.

An analysis to determine the complex interaction of the disease variables was done by fitting the data to a tree model (Figure 67). The results showed that lesion width was by far the most important variable that accounted for the observed measurements of necrosis within the stem. When lesion width was less than 0.7 mm, lesion length was the next most important variable (Figure 67). When both lesion length and lesion width were small, the proportional stem cross sectional area with necrosis was five percent. When lesion width was high and lesion length was low, the distance of colonisation (spread) became important.

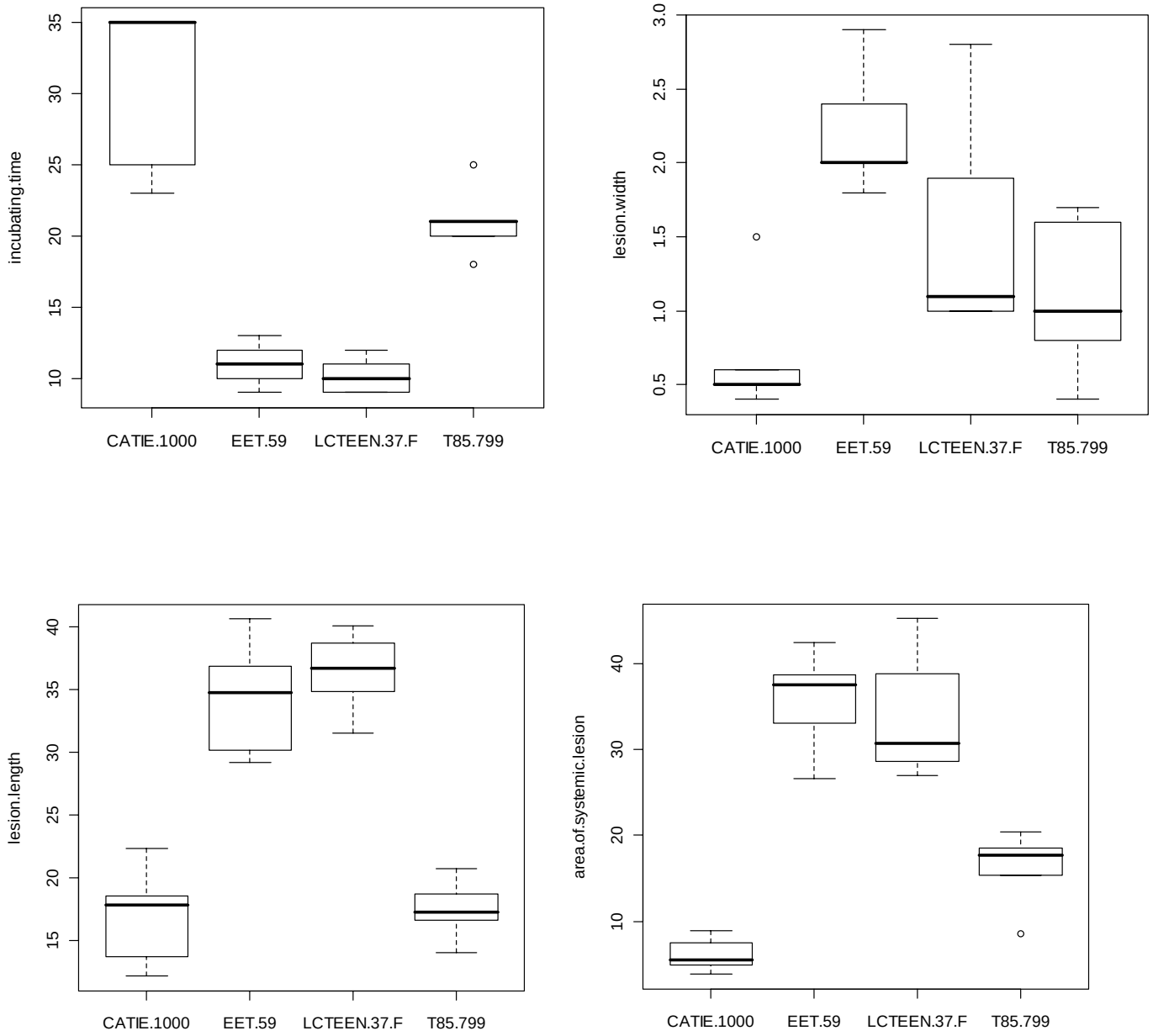

Figure 58. Box and whisker plots of selected resistance variables of selected clones (CATIE 1000, EET 59, LCTEEN 37/F and T85/799) showing the mean, 25th, 75th percentiles and non-outlier maximum and minimum- (inoculation with *L. theobromae*).

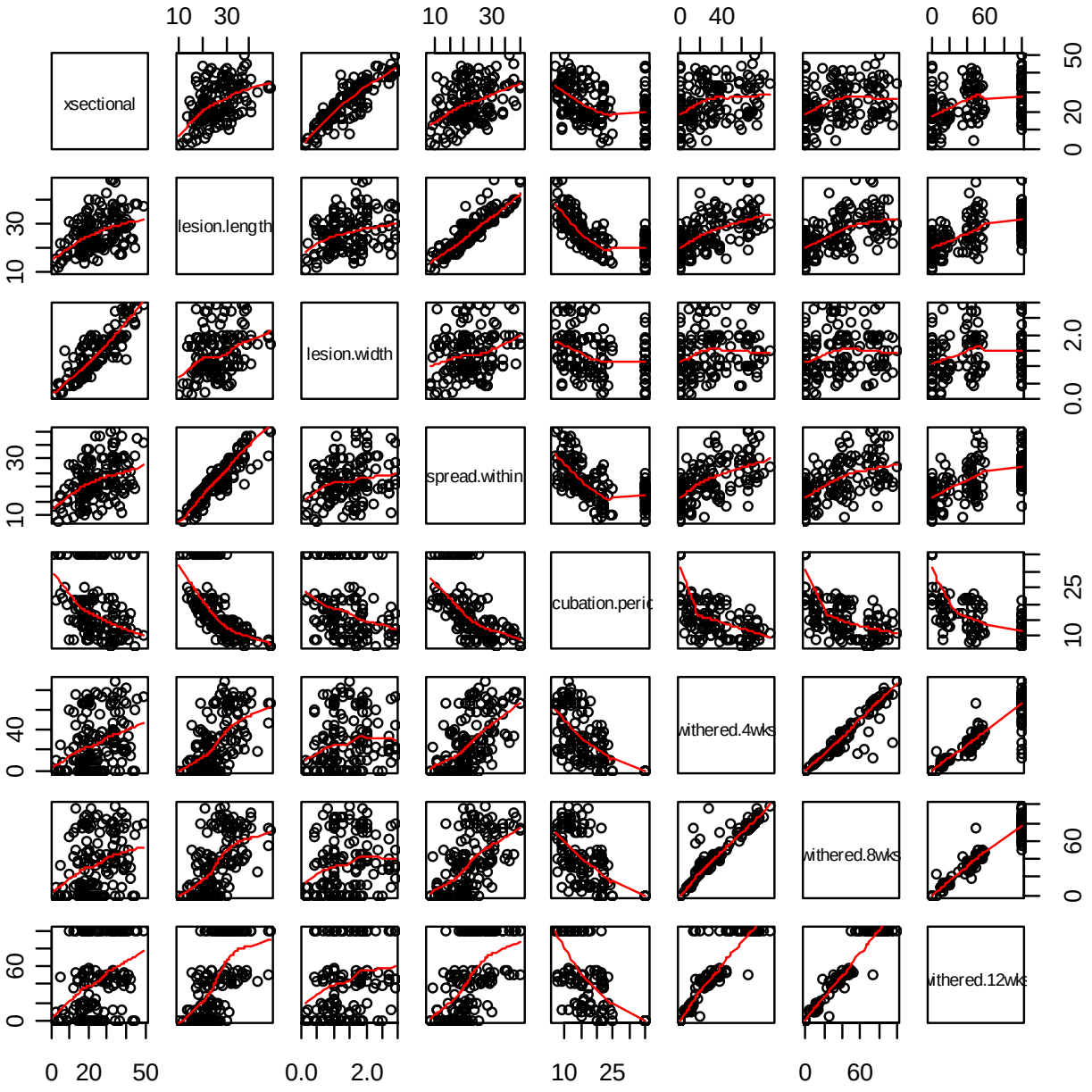

Figure 59. Relationships between variables used to evaluate resistance performance of cocoa germplasm to *F. decemcellulare and L. theobromae* studied in the greenhouse and for dieback disease in cocoa clones. Xsectional = percentage necrotic area of stem; lesion.length = lesion length; lesion.width = lesion width; spread.within = fungal colonisation of stem; cubation.peri = incubation period; withered.4wks = proportion of withered leaves 4 weeks after inoculation; withered.8wks = proportion of withered leaves 8 weeks after inoculation; withered.8wks; withered.12wks = proportion of withered leaves 12 weeks after inoculation; withered.8wks.

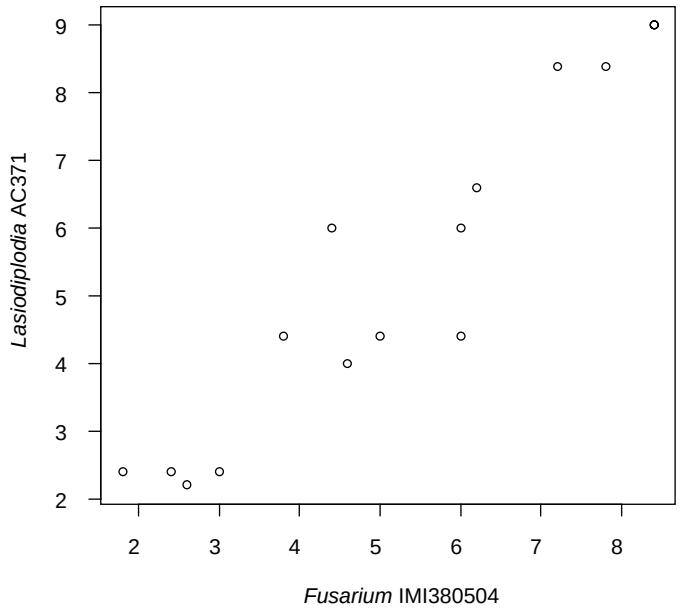

Figure 60. Pearson's product-moment correlation of dieback scores by *Lasiodiplodia pseudotheobromae* AC371 and *F. decemcellulare* IMI380504 on different cocoa germplasm lines. r = 0.9, t = 10.7629, df = 13, p-value = 7.6188-08; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from 0.8477011 to 0.9829978.

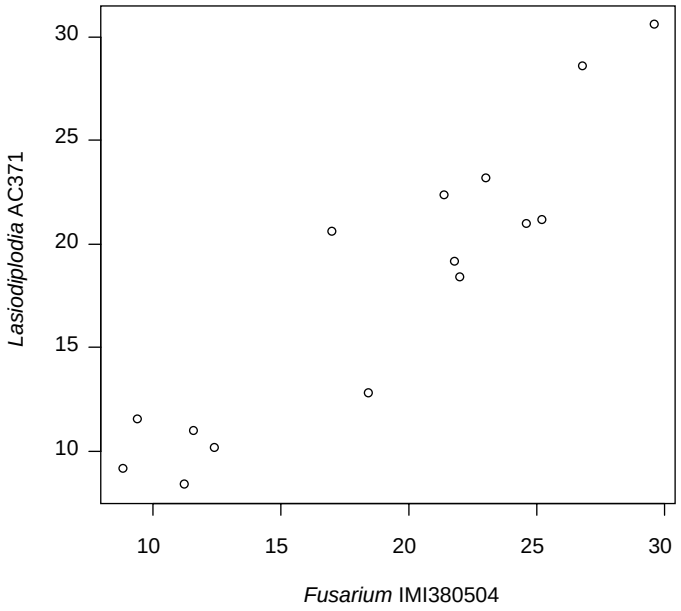

Figure 61. Pearson's product-moment correlation of incubation period by *Lasiodiplodia pseudotheobromae* AC371 and *F. decemcellulare* IMI380504 on different cocoa germplasm lines. r = 0.9, t = 8.7386, df = 13, p-value = 8.396e-07; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from 0.7828533 to 0.9749783.

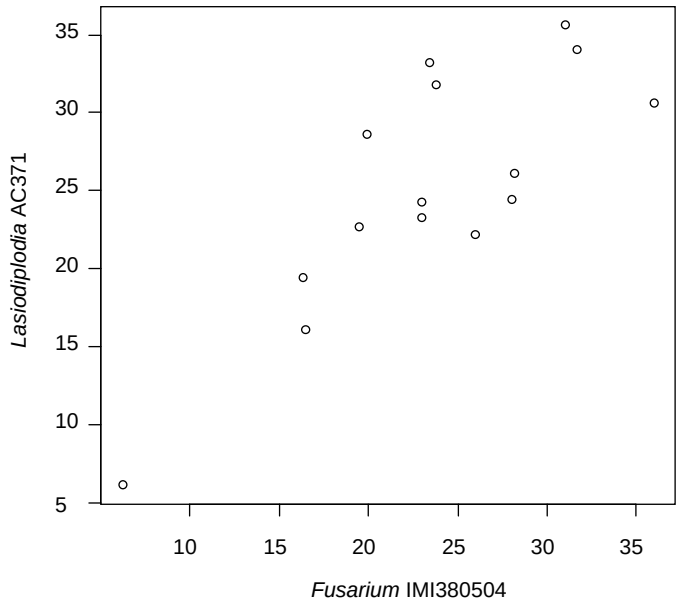

Figure 62. Pearson's product-moment correlation of percentage stem necrosis by *Lasiodiplodia* isolate AC371 and *F. decemcellulare* IMI380504 on different cocoa germplasm lines. r = 0.8, t = 5.0282, df = 13, p-value = 0.0002312; alternative hypothesis true; correlation is not equal to 0; 95 % confidence interval ranges from 0.5146415 to 0.9354851.

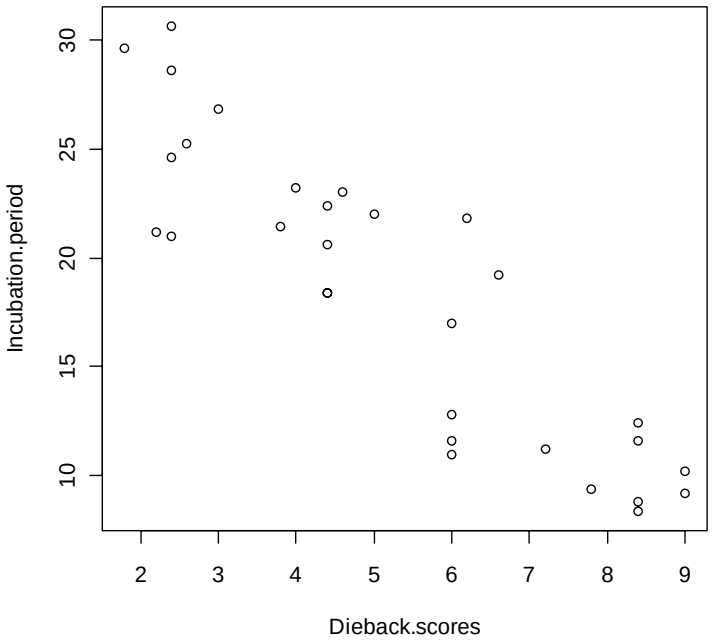

Figure 63. Pearson's product-moment correlation of incubation period versus dieback scores by *Lasiodiplodia* isolate AC371 and *F. decemcellulare* isolate IMI380504 (pooled data) on different cocoa germplasm lines. r = -0.9, t = -10.5149, df = 28, p-value = 3.141e-11; alternative hypothesis true; correlation is not equal to 0; 95 % confidence interval ranges from -0.9483432 to -0.7859186.

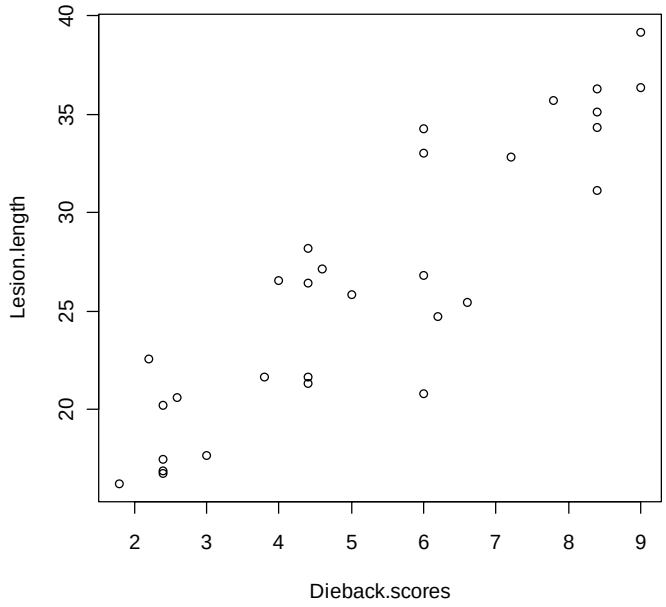

Figure 64. Pearson's product-moment correlation of lesion length versus dieback scores by *Lasiodiplodia* isolate AC371 and *F. decemcellulare* isolate IMI380504 (pooled data) on different cocoa germplasm lines. r = 0.9, t = 10.5416, df = 28, p-value = 2.967e-11; alternative hypothesis true; correlation is not equal to 0; 95 % confidence interval ranges from 0.786784 to 0.948571.

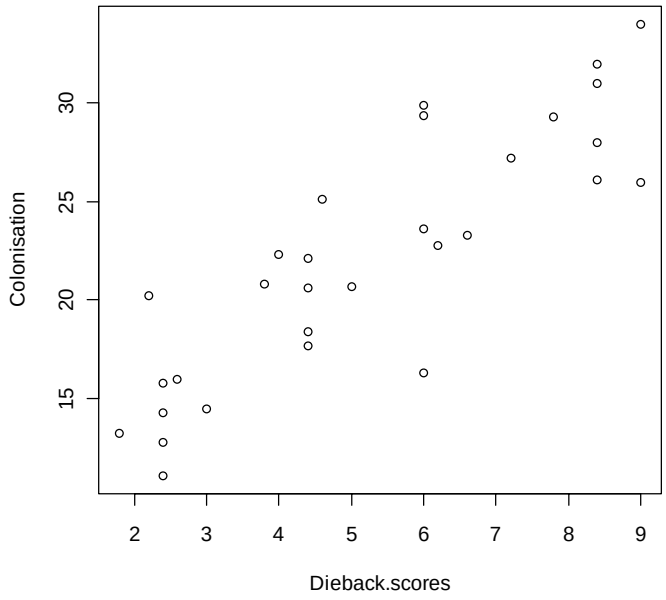

Figure 65. Pearson's product-moment correlation of colonisation versus dieback scores by *Lasiodiplodia* isolate AC371 and *F. decemcellulare* isolate IMI380504 (pooled data) on different cocoa germplasm lines. r = 0.8, t = 8.9593, df = 28, p-value = 1.027e-09; alternative hypothesis true; correlation is not equal to 0; 95 % confidence interval ranges from 0.7259651 to 0.9321486.

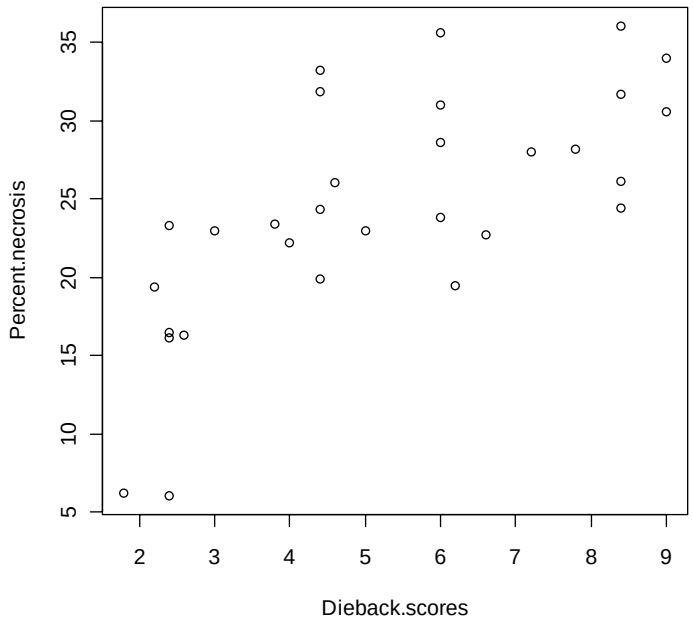

Figure 66. Pearson's product-moment correlation of dieback scores vs. percentage stem necrosis by *Lasiodiplodia* isolate AC371 and *F. decemcellulare* IMI380504 on different cocoa germplasm lines. r = 0.7, t = 5.0807, df = 28, p-value = 2.224e-05; alternative hypothesis; true correlation is not equal to 0; 95 % confidence interval ranges from 0.43428178 to 0.8426144.

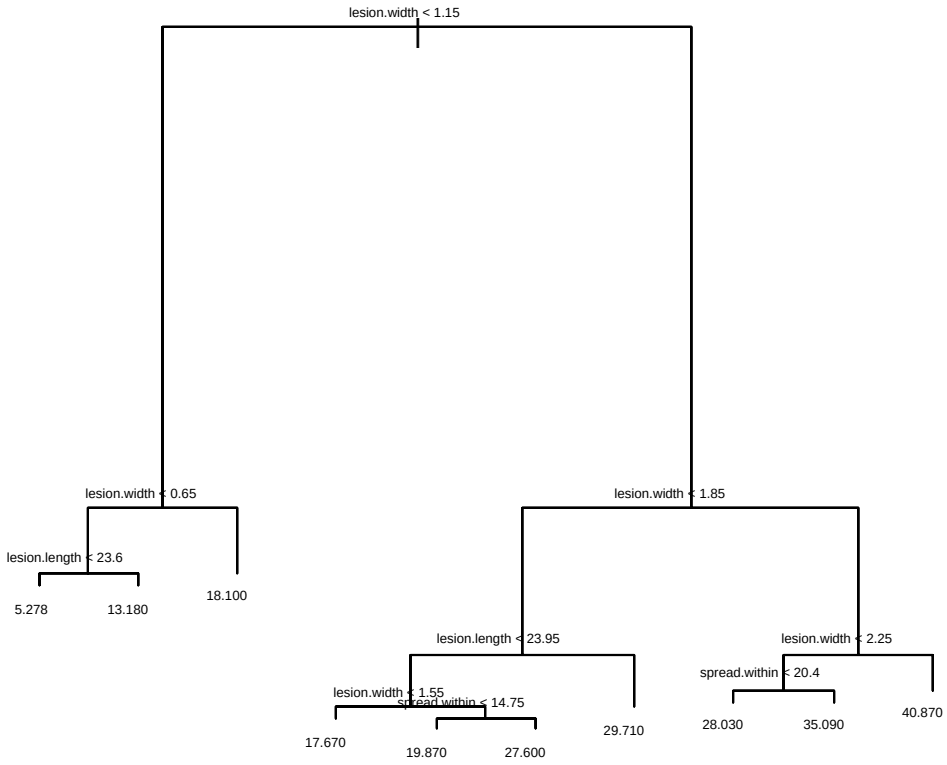

Figure 67. An illustration of how the different explanatory resistance variables influenced percentage necrotic area of stem (when the latter was used as a main response variable) using generalised additive model (gam) for the determination of those variables that significantly affected stem necrosis and, therefore, dieback in cocoa. The lengths of branches are proportional to explained variation in the response variable. Figures at the ends are the mean response values. If lesion width < 1.15 but > 0.65, the response variable is 18.1. If lesion width > 2.25 the response variable is 40.87. Spread within only matters if lesion width is > 1.15 but < 1.85. Lesion width is by far the most important explanatory variable.

4.4.4.5 Comparison of stem lesion and colonisation in laboratory and greenhouse studies

Results for the two pathogens, isolates AC371 and IMI380504 from the greenhouse studies were analysed separately even though they were not significantly different ($P > 0.05$). Possibly due to differences in the length of time the experiments were run or other unknown factors, all across-clone and all across-isolate mean lesion sizes were smaller in the laboratory detached stem trial compared with the greenhouse experiment (Table 20 and Table 21). Inoculation with isolate AC 371 caused

significantly longer lesions than isolate IMI 380504 in the laboratory, but in the greenhouse, their effects were similar.

4.5 Discussion

Screening for sources of resistance to diseases is the first step in a genetic improvement-breeding programme. The aim of the present study was to investigate differences in the host–pathogen interaction between cocoa and its principal dieback pathogens, *F. decemcellulare* and *L. theobromae.* To gain an insight into whether the differences in resistance among the different clones reflect natural field infection, greenhouse-budded plants were wound inoculated with spore suspension of isolates of the two fungi. The germplasm lines used in the laboratory and greenhouse are given in Table 14 and Table 16 according to their responses to the isolates investigated. Symptom development in stem segments and whole plants was observed from the initial infection through to the appearance of signs of foliar dieback, and finally death of the plant. The laboratory and greenhouse resistance screening methods described in the present investigation of detached stems as well as whole plants provides a double opportunity of identifying resistance in cocoa germplasm.

The lack of suitable criteria for interpretation of resistance was recognised earlier (Owen, 1956), hence in the present study several resistance components were used for determining overall response in the greenhouse. Unlike past evaluation methods that measured only lesions, the current method has the advantage of potential scale-up so it can be applied to large sample of clones. Moreover, these methods can be performed in a standardised way so they can be repeated regularly, giving a measure of the trend in the resistant plants. In the current study, a multivariate analysis of the resistance components was carried out, enabling correlations among them to be assessed. The laboratory and greenhouse resistance screening methods were simple but the results were inconsistent. However, before they could be applied on a broader scale for the rapid selection of dieback-resistant clones, they would need to be validated by field testing in a range of tropical environments. The correlation of pathogenicity between the different isolates was investigated. In the laboratory test, a correlation between the ability to colonise and induce necrosis by *Lasiodiplodia* isolate AC845 and *F. decemcellulare* IMI380504 or *Lasiodiplodia* AC371 was not

straightforward. There is a significant relationship, however, between colonisation and the induction of necrosis by *F. decemcellulare* isolate IMI380504 or *Lasiodiplodia* isolate AC371 (Figure 47 and Figure 50). Many of the graphs appear to show outlier points (i.e. values in the dataset positioned at unusually large distances from the rest). These points look very similar in corresponding graphs. However because the data of the measurements of lesion and the distance of spread are normal, these 'outliers' may be linked to the response of particular clones e.g. GU 123/V and not necessarily to non-normality.

The various clones showed differences in response to the two fungal isolates in terms of both the sizes of necrotic lesions and the degree of tissue colonisation. In the greenhouse test to compare necrotic symptoms and dieback, the two isolates (*F. decemcellulare* IMI380504 and *Lasiodiplodia* isolate AC371) produced similar symptoms. As noted in Chapter 2, the results showed slight differences in the pathogenicity between the isolates, which was consistent over the two trials i.e. in the laboratory and in the greenhouse, but the basis for this variation was not established. These fungi presumably produce toxic metabolites within the host tissue that may have a role in the expression of dieback symptoms.

The infection of cocoa by both fungi was characterised by progressive degradation and death of the woody tissue and this was followed by foliar symptoms that developed within 25 days of inoculation. The action of the pathogens was consistent on the different clones and probably the development of dieback symptoms was preceded by symptomless infection characterised by the formation of dark brown necrotic lesions in the stem tissues.

In the laboratory detached stem evaluation, all the cocoa clones developed visible and measurable necrotic lesions. There was none without necrotic lesions among the 29 clones tested under conditions of the study. Symptoms consisted primarily of necrotic lesions near the point of inoculation, and a brown band of colour along the vertical axis of the stem, where the fungus may have spread in the tissue. Necrotic lesions developed throughout the stem, but commonly downward from the point of inoculation. The results show considerable variation in response among the different cocoa germplasm lines studied.

The average size of lesions in the different cocoa stem segments was larger than has previously been reported (Owen, 1956). The larger sizes of the lesions found in the present study may be related to the age of the budded plants (three months), which was younger than the seedling age reported in the infection studies by Owen (1956) of two to three years. On the other hand, the necrotic lesions in this study had a similar elliptical shape to those reported by Crowdy (1947) and Owen (1956). Large necrotic lesions did not develop in some clones for example NA 149 against IMI380504, but the plants did show substantial colonisation. Table 18 and Table 21 indicate those genotypes with good performance to *Lasiodiplodia* and *Fusarium* infections, SCA 6 was the most 'resistant' detached clone. SCA 6 could have common resistance genes controlling both necrotic lesion development and stem colonisation. The results of the *in-vitro* studies suggest the possibility of identifying cocoa genotypes with adequate level of resistance to both *Lasiodiplodia* and *Fusarium* infections. Further experiments are needed to evaluate the repeatability of the results.

In the greenhouse evaluation, one of the variables investigated was the internal infection of the different clones as assessed by the extent of necrosis as well as tissue colonisation by the fungus. Systemic infection consisted primarily of necrotic lesions near to the point of inoculation, and a brown band of colour and brown patches along the vertical axis of the stem, where the fungus may have spread in the tissue. Necrotic lesions developed throughout the stem, but commonly downward from the point of inoculation. However, the study demonstrated that the greenhouse inoculation method, which incorporated an estimate of dieback severity among other things, revealed clonal resistance differences more clearly than those which assessed only lesion size and stem colonisation. This apparent conflict may be resolved when considering the fact that the activation of defense reactions by some plants can presumably result in the production of biologically active and toxic compounds that can cause host cell destruction, necrotic responses, and tissue death.

The *in-vitro* laboratory studies have shown that the three isolates varied both in their ability to cause lesions and colonize different cocoa genotypes. The two assessment methods employed probably measured different resistance factors, and it may be that resistance factors which control tissue death are different from those controlling

fungal progression in the plant tissue. Though it was found that there was a wide range of tolerance among the genotypes, it is too early at this point to draw conclusions about the factors involved in limiting fungal spread in the tolerant genotypes.

As some isolates were not sufficiently aggressive to differentiate between cocoa genotypes, it appears important to take into consideration both aggressiveness and diversity of isolates for testing *Lasiodiplodia* and *Fusarium* stem canker resistance in cocoa *in-vivo*.

Table 20 and Table 21 show that the distance of spread by *F. decemcellulare* and *Lasiodiplodia* in the stem tissue was in the range 15 to 20 mm on Amelonado and LCTEEN 37/F, whereas in CATIE 1000 pathogen growth was limited to 5 and 10 mm. In the assessment carried out 6 weeks after inoculation, the distance by which the fungi had spread up the plant from the point of inoculation was 5 mm in Amelonado, EET 59, LCTEEN 37/A, LCTEEN 37/F and none in CATIE 1000, T85/799 and MXC 67. In the susceptible clones such as Amelonado, LCTEEN 37/A, LCTEEN 37/F, LCTEEN 37/G and PNG 418, the rate of fungal spread within the stem was faster than in the resistant clones such as CATIE 1000 and T85/799. Results from this study indicated that stem colonisation can lead to dieback symptoms as there was a positive correlation between the two variables. It was evident from these results that both fungi induced dieback by comparable amounts as shown by the correlation coefficient values (Figure 60 to Figure 62), and therefore, control strategies developed against either of the two pathogens may be able to perform to the same measure against the other. While the results reported here suggest a relationship between ability of both fungi to induce disease, it will be necessary to analyse and study more isolates as they become available.

Owen's (1956) method of determining susceptibility of cocoa types to *F. decemcellulare* was limited because he only assessed necrotic lesions to determine resistance. The method described here analyses the level of resistance in the different clones by measuring necrotic lesions as well as dieback symptoms, internal colonisation and incubation period. The reaction of the different cocoa clones to *F. decemcellulare* or *Lasiodiplodia* differed under the greenhouse conditions and there

were a few replicates that showed no dieback disease symptom. The implication of this observation will require further work as these plants may have escaped infection. The traditionally cultivated West African Amelonado variety and some of the newer clones were the most susceptible to dieback and symptoms closely reflect natural field infection. The one asymptomatic Amelonado control in the *F. decemcellulare* group was most probably an escape caused by reduced fungus inoculum received by the plant. When necrotic lesions, which have so far mostly been used, were compared with foliar symptoms based on visual observations, a clear distinction between susceptible and resistant interactions were observed. The incubation periods and fungal colonisation were also good indicators for assessing resistance in the different cocoa genotypes as there is a linear relationship between them and the internal stem necrosis. To avoid the limitations of earlier studies that were based on undefined mixtures of fungal genotypes, a large number of cocoa accessions were here screened with single conidial isolates. The use of single-conidial isolates is important to avoid mixed host responses resulting from combinations of compatible and incompatible interactions.

The variability in tolerance will be influenced by host plant genetics and other unexplained factors. Numerous investigations on the resistance of cocoa to fungal diseases have been reported in the literature. Capriles *et al.* (1964), Capriles and Reys (1968), Brownlee *et al.* (1990), Cooper *et al.* (1995) and Resende *et al*. (1996) investigated different compounds produced during infection in cocoa, and concluded that the amounts of phenolic compounds, tannins, polymeric procyanidins and elemental sulphur were part of the plant's response to infection and contributed to inhibition of disease progression. Nojosa and colleagues (2003) showed that oxidative enzymes influenced cocoa genotypes' resistance to the frosty pod pathogen, *Crinipellis perniciosa*. Likewise, ongoing research on cocoa by Verica and Maximova (2004) have shown that defence responses are activated via endogenous signalling molecules such as salicylic acid, jasmonic acid and ethylene through changes in the expression of a number of genes. Thevenin *et al.* (2005) suggested that witches' broom resistance in some cocoa genotypes was most likely physiological, and broom development was possible when the point of infection was near to the meristematic region. Many studies including those of Evans and Bastos (1975) and Danquah (1986) have shown that basidiospores have slower germ tube growth in extracts from SCA 6

than extracts from susceptible germplasm. Surujdeo-Maharaj and Umaharan (2004), working with fourteen cocoa germplasm lines (SCA 6, SCA 12, ICS 1, ICS 84, ICS 95, IMC 57, IMC 67, JA 6/4 [POU], JA 5/19 [POU], JA 5/41 [POU], UF 11, Hybrid 19, M 8, and West African Amelonado) also reported a decline in witches' broom development in the SCA and IMC clones through delayed colonisation of meristematic tissues.

Results from the current study indicate that the two fungi tested have a similar ability to cause disease and that fungal growth was slow and establishment within stem tissue was low in resistant clones. Where attack was successful, host reaction may have acted to slow disease expression, giving an increased incubation period and slowing the development of necrotic lesions and dieback. The components of resistance evaluated in the current study were grouped in five categories, each related to a different aspect of response to disease: longitudinal spread of necrotic lesions, lateral spread of lesions, incubation period, distance of spread of viable hyphae and dieback severity scores. In addition, significantly delayed symptom development was demonstrated by a decrease in the disease severity scores of the resistant clones by a factor of about 60 % compared with the susceptible clones ($P \leq 0.05$) (Table 20 and Table 21). Thus, in the susceptible clones (e.g. LCTEEN 37/F) the foliar symptom of dieback was detected from the tenth day whereas in the resistant clone T85/799, foliar symptoms became observable only after 20 days.

The results also showed a significant linear relationship between visual estimates of dieback and lesion size. Clones with large lateral necrotic lesions tended to be more susceptible to dieback, whereas clones with small lateral lesions showed some 'resistance'. It is of great importance that in cocoa germplasm a certain correlation can be demonstrated between resistance to dieback and other easily determinable variables such as lateral lesion size. Visual symptoms have the advantage of non-destructive assessment and can be made progressively over time. Their positive correlation with the internal criteria of pathogen progress is reassuring.

Responses of cocoa germplasm to dieback disease have been evaluated in two ways: using detached stem segments in the laboratory, and intact plants in the greenhouse. In view of the fact that the laboratory stem-segment experimental methods were

reproducible but gave results inconsistent with the greenhouse whole-plant inoculation methods (Figure 56). The results suggest that some cocoa germplasm lines retard the development of *F. decemcellulare* and *L. theobromae* in the plant but the question of mechanism of resistance remains unanswered. The search for resistance has paid off with a promising result in that some dieback-resistant plants have been identified among the cocoa genotypes studied. A breeding programme to improve the level of dieback resistance in cocoa can be successful by discarding all relatively susceptible clones and direct selection for resistance under field conditions. Further screening of CATIE 1000 and T85/799 is warranted for field use and possibly for control of dieback disease in West Africa. The expression of 'resistance' in only a few but not all the clones studied indicates a need for further studies on a larger number of clones for disease resistance.

In the present study, plants were inoculated once only, at a single point, and at a young stage of growth, contrasting with a real cocoa farm in which multiple inoculations occur through mirid feeding punctures. Although the results demonstrate that differences in plant response exist among the different cocoa germplasms, the range of values for disease obtained in this trial may not adequately reflect what is possible under field conditions. Nine of the ten plants budded with CATIE 1000 material, which was maintained in the greenhouse, survived to the end of the year. The report from Capriles *et al.* (1964) about the high resistance of clone IMC 67 to *Ceratocystis fimbriata* correlates weakly with its response to the pathogens used in the current study, emphasising the importance of knowing precisely which pathogen one is dealing with.

In summary, twenty-nine and fifteen lines of cocoa germplasm were screened in the laboratory and the greenhouse, respectively, for resistance to isolates of *F. decemcellulare* and *L. theobromae.* The laboratory screening method lasted three to five weeks less than the greenhouse testing (including time for plant propagation, inoculation, and assessment and used less than 10 percent of the space required for greenhouse plants. Moreover, fourteen more clones were tested in the laboratory than were tested in the greenhouse. Though *in-vitro* screening of dieback-resistant plants can only augment eventual greenhouse and field testing, this report suggests that it can be an efficient initial selection method, which could significantly reduce the time

and cost of plant selection and breeding. Furthermore, *in-vitro* methods may have utility in the screening of resistance to other diseases (e.g. black pod). The use of stem segments in this study provided a simplified experimental system that allowed the testing of a large number of clones with results that correlated well with the reactions given by whole plants. At the other end of the scale, field-grown plants would involve a long period of waiting for plants to mature, would be more expensive and open to the possibility of pest infestation and complications from other diseases. Though the typical dieback disease symptoms seen on infected plants grown in the greenhouse were not obvious on detached stems infected *in-vitro*, measurement of lesions and detection of fungi by culturing was reproducible for both situations and represents an objective testing method. Greenhouse inoculation of the clones did appear to produce bigger lesions than laboratory inoculation, but this is probably due to the longer period of exposure in the greenhouse.

Genotypic differences were found in both tests among the different cocoa germplasms studied and the clones showed a wide range of disease reactions from nearly resistant to very susceptible. The pathogenicity of *F. decemcellulare* IMI380504 and *Lasiodiplodia* AC371 were found to be similar in this study which suggests that a breeding programme for controlling one of the pathogens can have benefit against the other. Direct significant correlations ($r = 0.7$) ($P \leq 0.01$) were obtained between visual dieback assessment scores and the percentage of stem necrosis (Figure 66). In addition, the response of inoculated stem segments corresponded to the reaction of budded intact plants to *F. decemcellulare* and *L. theobromae.* On the basis of the aggregate results, the study identified three clones (i.e. CATIE 1000, T85/799 and MXC 67) with high levels of dieback 'resistance' that could act as donor germplasm in a programme of genetic improvement. It is unlikely that the resistant clones detected here could be used directly by farmers as the reproducibility of these results would require validation in field trials. Detailed field testing, therefore, is needed to provide an absolute assurance for their use.

5. Chapter 5

5.1.General discussion and conclusions

Theobroma cacao L. has a rich history of human use dating from the ancient Olmec and Mayan folks of the Eastern Mexican gulf (Dillinger *et al*., 2000; Bennett, 2003). Over the centuries, however, the earlier medicinal uses of cocoa have declined until current research established the role of cocoa in promoting cardiovascular health (Kris-Etherton and Keen, 2002). This is allegedly dependent on certain polyphenols (catechins and flavanols) that are potent antioxidants that can ease inflammatory processes in atherosclerosis by blocking expression of cellular adhesion molecules.

The cocoa plant received increased attention towards the end of the nineteenth century because of its high yield of pods, which are rich in juice, aroma, and butter, and because of its presumed potential for cultivation with organic production methods (Amoah, 1995). In the 1930s, an extensive collection of cocoa germplasm was made in the Peruvian Amazon region (maintained in Trinidad) in order to provide a wide genetic resource (Pound, 1938). But commercial cocoa still has a rather narrow genetic base as there has been little use of the germplasm for conventional breeding, which is why cocoa production in the state of Bahia, Brazil was ruined in the 1990s by witches' broom disease (Bennett, 2003). Thus, the crop has been left exposed in the face of these assaults, by not incorporating resistance and effective defence mechanisms to prevent or limit infection.

In West Africa where about 70 % of the world cocoa is produced, numerous factors limit cocoa production including a wide array of diseases and abiotic stresses. Significant financial losses due to black pod and dieback diseases continue to discourage farmers from going to the land (Amoah, 1995). Additionally, insect pests such as mirids, stinkbugs, and stem borers affect production and necessitate the use of conventional commercial pesticides (Adu-Acheampong, 1997; Adu-Acheampong and Ackonor, 2005). The frequent application of insecticides needed to control mirids that are associated with dieback might be reduced if clones with sufficient levels of resistance become available, together with husbandry practices that discourage them.

Given that the microbial causes of dieback have remained obscure until now it is hardly surprising that there has been little progress in understanding its epidemiology or in selecting cocoa germplasm for resistance. The following conclusions can be drawn from the current investigation:

- Morphological and molecular methods showed the presence of four *Fusarium* and two *Lasiodiplodia* species in dieback lesions. *Fusarium decemcellulare* was not isolated in the course of the study, contradicting established literature on the subject.
- All of the *Lasiodiplodia* isolates were able to cause dieback, whereas 44 of the isolated *Fusarium* isolates could not. Some of these non-pathogenic organisms deserve further study for potential use in biological control programmes.
- Examination of freehand-cut sections by light microscopy did not show fungal hyphae but stems dissected for evaluation of fungal attack and plated onto agar revealed the extent of colonisation.
- Duration of symptoms prior to tree death was considerably shorter in the susceptible group of cocoa clones and this group was more likely to shed their leaves. These observations resemble the field symptoms of dieback disease and this may be the true experimental picture of *Fusarium* spp. and *Lasiodiplodia theobromae* infection.
- There was no significant correlation ($P < 0.05$) between *in vitro* and greenhouse results of screening for resistance. The results from this study showed that the *in vitro* test was only suitable for selecting very resistant genotypes and, therefore, will only serve as an initial selection method.
- There was a positive correlation between dieback scores and both visible lesion length and extent of fungal colonisation ($P < 0.001$). Any of these would be a suitable criterion for future evaluation of resistance of cocoa genotypes.
- CATIE 1000, MXC 67 and T85/799 exhibited longer incubation periods and lower dieback scores than the other germplasm lines and it would be interesting to see whether they would maintain this position under field conditions.

5.1.1. Recommendations for future research direction

Significant control of dieback disease of cocoa will most likely depend on resistant clones developed through conventional breeding using as partial parentage those plants that consistently express resistance against the causal pathogens. Should research be intensified in this direction in the medium term, the identification of highly resistant clones will not only contribute to reducing dieback, but also may contribute to control of other fungal disease-causing agents. It is likely that resistant cocoa clones could be disseminated very rapidly across West Africa, driven by farmers' demand for disease control methods that will reduce exposure to hazardous pesticides and incidences of poisoning. The indication is that the use of resistant cocoa will be extremely valuable to the over 800,000 smallholder farmers in Ghana. They will be able to increase their yield per ha, reduce pesticide costs, and thereby increase their income. The current situation where farmers purchase their planting materials from the Seed Garden Unit of the Ghana Cocoa Board (COCOBOD) gives hope that farmers will make the informed decisions to adopt clones based on their assessment of the costs and benefits. As they find it profitable, we would expect cocoa farmers on small farms in many other West African countries to achieve similar gains, especially in the Ivory Coast, Nigeria, and the Cameroon where cocoa farmers face the same mirid-fungi-dieback problem, and where mirids have become resistant to many of the most common pesticides. The foregoing, including the discussion regarding the research's future prospects, contains certain forward-looking statements that involve hopes for the farmer with limited resources.

To this end a research proposal is outlined for screening of cocoa germplasm lines against dieback disease.

Proposals for future work

Goals:

- To increase our understanding of the morphological, physiological, genetical, and/or biochemical basis of resistance to *Fusarium* and *Lasiodiplodia*-associated dieback in cocoa genotypes and to transfer the identified resistance into clones possessing good quality traits such as good chocolate flavour, desirable aroma, larger bean size and desirable agronomic characteristics.

- To develop clones with durable resistant genes effective under West African conditions to sustain rural cocoa farming by reducing frequency of pesticide spraying, increasing yield, and improving the green vegetation cover and conditions in the dry season.

Rationale and significance

Cocoa (*Theobroma cacao* L.) is the most widely cultivated cash crop in West Africa and its economic significance is comparable only to minerals and timber. Dieback disease causes yield losses of 25 to 30%. However, on young cocoa trees, and under conditions favourable for disease development, losses can be higher than 60 % (Owusu-Manu, Personal communication). This is coupled with significant delay in the time to fruiting. Most of the commonly grown hybrid cultivars are susceptible to dieback, hence the importance of developing resistant germplasm lines. The major objective of this study is to identify the genetic basis of resistance effective against dieback and transfer it into existing elite cocoa genotypes. The germplasm so developed will be evaluated for agronomic and quality traits and released as improved cultivars to be grown in Ghana.

Approaches:

- National survey to sample and characterise fungi associated with dieback of cocoa.
- Collection and characterisation of cocoa genetic resources.

- Laboratory, greenhouse and field inoculation tests coupled with comparison between natural infections and artificial inoculations.
- Breeding: employing DNA-based crop selection techniques, interspecific hybridizations and appropriate cytogenetic manipulations.
- Participatory varietal selection.

Relationship to present work and research plan:

In this thesis, the reaction of a number of international cocoa germplasm lines kept at the University of Reading, UK were evaluated. Few genotypes showed resistant reactions under greenhouse conditions but three clones (CATIE 1000, MXC 67 and T85/799), were found to be good sources of resistance to dieback disease. The Amelonado cultivar was susceptible and a number of *Fusarium* isolates collected from field infected dieback lesions were non pathogenic. It is hereby proposed that a national survey is conducted to collect fungi associated with dieback in all cocoa growing regions of Ghana and elsewhere in West Africa for further screening. Additional laboratory and greenhouse inoculation tests will be conducted on a diverse germplasm collection (from the Reading stock) from which promising genotypes will be selected. Detailed studies on the infection process of *Fusarium* and *Lasiodiplodia* will be carried out by biochemical, molecular and ultrastructural techniques to understand the mechanism of resistance. Subsequently, *in-vitro* and greenhouse results will be validated in field trials. Hybridisation and other methods of crop improvement will be employed to increase the genetic base of the resistant plants and combine resistance with desirable quality traits and agronomic characteristics. The newly identified sources of resistance will be graded, hybridised with superior germplasm and tested in greenhouse and inoculated-field tests. Previously identified molecular markers linked to other resistance genes in cocoa (Meyers *et al*., 1998) would be used to confirm the presence of resistance genes in these lines. The resistant lines will be screened in the field in multi-location facilities and artificially inoculated. The field screening will also give opportunity for evaluation of agronomic performance of these genotypes. Superior germplasm lines that combine high yield, quality and multiple disease resistance genes will be released to be grown by farmers to improve the economic efficiency of cocoa production.

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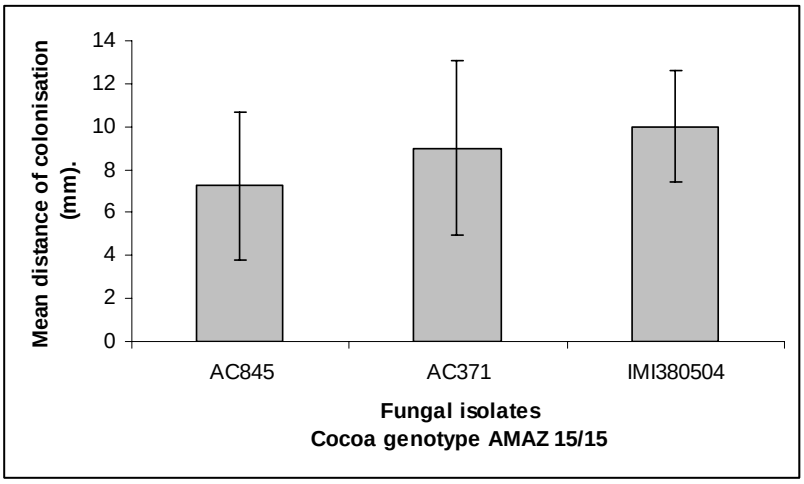
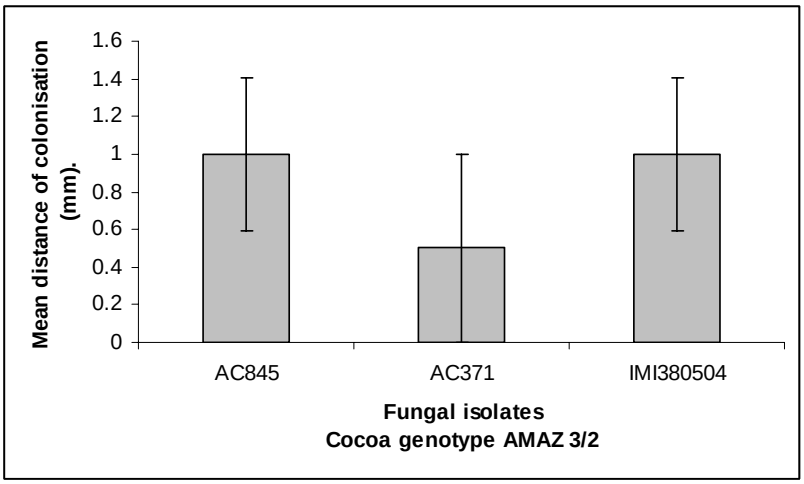
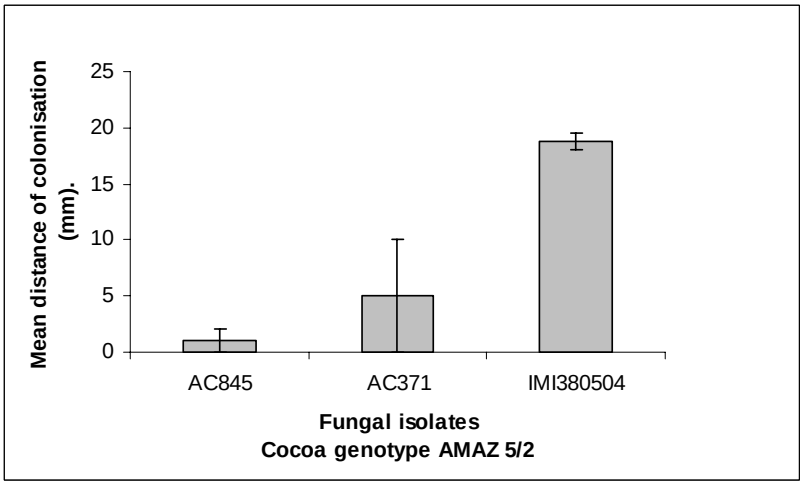
Appendix 1. Linear growth rate (mm per day) of isolates of F. decemcellulare, Lasiodiplodia theobromae and Fusarium spp. isolated from cocoa. Values within columns followed by the same superscript letter do not differ significantly at P ≤ 0.05).

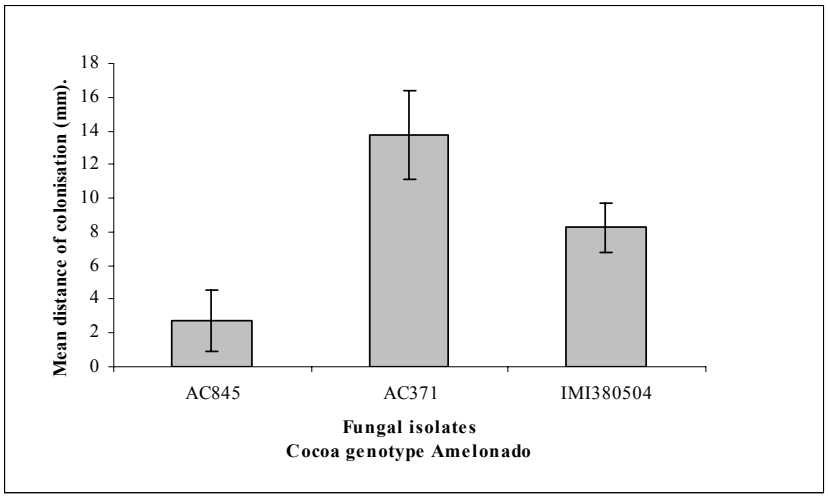
Fungus	Isolate	Mean radial growth rate of colonies (mm per day) [x]					
		10˚C	15˚C	20˚C	25˚C	30˚C	35˚C
F. decemcellulare	IMI 380504	0.0	0.5[a]	0.8[a]	1.3[a]	1.5[a]	1.0[a]
	IMI 361352	0.0	0.5[a]	0.8[a]	1.3[a]	1.5[a]	0.6[a]
	GJS 03-81	0.0	0.6[a]	0.8[a]	1.4[a]	1.8[a]	0.6[a]
	GJS 01-170	0.0	0.5[a]	1.0[a]	1.5[a]	1.5[a]	1.0[a]
Fusarium species	AC 037	0.0	0.5[a]	1.0[a]	1.4[a]	1.5[a]	1.0[a]
	AC 110	0.0	0.5[a]	1.0[a]	1.3[a]	1.6[a]	1.2[a]
	AC 120	0.0	0.5[a]	1.1[a]	1.3[a]	2.0[a]	1.3[a]
	AC 174	0.0	0.6[a]	1.2[a]	1.5[a]	1.5[a]	0.9[a]
	AC 197	0.0	0.5[a]	1.2[a]	1.6[a]	2.0[a]	1.2[a]
	AC 206	0.0	0.5[a]	1.2[a]	1.5[a]	1.8[a]	1.0[a]
	AC 229	0.0	0.6[a]	1.1[a]	1.4[a]	1.7[a]	1.2[a]
	AC 255	0.0	0.5[a]	1.2[a]	1.4[a]	1.8[a]	1.3[a]
	AC 276	0.0	0.5[a]	1.2[a]	1.5[a]	1.8[a]	1.2[a]
	AC 284	0.0	0.5[a]	1.2[a]	1.5[a]	2.0[a]	1.3[a]
	AC 328	0.0	0.5[a]	1.2[a]	1.5[a]	2.0[a]	1.3[a]
	AC 330	0.0	0.5[a]	1.2[a]	1.5[a]	1.8[a]	1.2[a]
	AC 402	0.0	0.6[a]	1.2[a]	1.4[a]	1.7[a]	1.2[a]
	AC 451	0.0	0.5[a]	1.2[a]	1.5[a]	1.8[a]	1.3[a]
	AC 593	0.0	0.5[a]	1.1[a]	1.3[a]	1.6[a]	1.0[a]
	AC 638	0.0	0.4[a]	1.3[a]	1.6[a]	2.0[a]	1.3[a]
	AC 656	0.0	0.5[a]	1.2[a]	1.5[a]	2.0[a]	1.3[a]
	AC 705	0.0	0.6[a]	1.2[a]	1.5[a]	1.9[a]	1.2[a]
	AC 773	0.0	0.6[a]	1.0[a]	1.2[a]	1.5[a]	1.1[a]
	AC 914	0.0	0.5[a]	1.0[a]	1.3[a]	1.6[a]	1.0[a]
	AC 317	0.0	0.5[a]	1.2[a]	1.5[a]	1.9[a]	1.2[a]
	AC 511	0.0	0.5[a]	1.0[a]	1.2[a]	1.5[a]	1.0[a]
	AC 787	0.0	0.5[a]	1.1[a]	1.3[a]	1.7[a]	1.2[a]
	AC 806	0.0	0.5[a]	1.2[a]	1.5[a]	2.0[a]	1.4[a]
	AC 031	0.0	0.5[a]	1.2[a]	1.5[a]	2.0[a]	1.4[a]
	AC 392	0.0	0.5[a]	1.1[a]	1.4[a]	1.7[a]	1.2[a]
	AC 748	0.0	0.5[a]	1.1[a]	1.4[a]	1.7[a]	1.2[a]
	AC 767	0.0	0.6[a]	1.0[a]	1.3[a]	1.6[a]	1.0[a]
	AC 076	0.0	0.5[a]	1.0[a]	1.4[a]	1.7[a]	1.0[a]
	AC 211	0.0	0.5[a]	1.0[a]	1.2[a]	1.5[a]	0.9[a]
	AC 219	0.0	0.5[a]	1.2[a]	1.6[a]	2.1[a]	1.3[a]
	AC 361	0.0	0.5[a]	1.2[a]	1.5[a]	2.0[a]	1.4[a]
	AC 551	0.0	0.5[a]	1.1[a]	1.4[a]	1.7[a]	1.2[a]
	AC 580	0.0	0.5[a]	1.1[a]	1.4[a]	1.7[a]	1.2[a]
	AC 739	0.0	0.5[a]	1.0[a]	1.2[a]	1.6[a]	0.9[a]
	AC 768	0.0	0.6[a]	1.0[a]	1.2[a]	1.5[a]	1.0[a]
	AC 995	0.0	0.5[a]	1.0[a]	1.2[a]	1.6[a]	0.8[a]
Lasiodiplodia species	IMI 333797	0.0	0.7[b]	7.3[b]	13.1[b]	14.8[b]	2.5[b]
	AC 008	0.0	0.8[b]	6.5[b]	12.3[b]	15.0[b]	2.3[b]
	AC 036	0.0	0.7[b]	6.6[b]	12.5[b]	14.7[b]	2.5[b]
	AC 064	0.0	0.8[b]	6.2[b]	13.0[b]	15.8[b]	2.4[b]
	AC 068	0.0	0.8[b]	7.0[b]	12.1[b]	14.4[b]	2.4[b]
	AC 280	0.0	0.8[b]	6.2[b]	12.0[b]	14.6[b]	2.4[b]
	AC 318	0.0	0.8[b]	6.0[b]	12.5[b]	14.7[b]	2.5[b]
	AC 322	0.0	0.8[b]	6.0[b]	13.0[b]	15.6[b]	2.4[b]
	AC 329	0.0	0.8[b]	6.2[b]	12.5[b]	14.7[b]	2.4[b]
	AC 360	0.0	0.7[b]	6.4[b]	12.0[b]	14.5[b]	2.5[b]
	AC 371	0.0	0.8[b]	6.5[b]	12.4[b]	14.7[b]	2.5[b]
	AC 375	0.0	0.8[b]	7.0[b]	12.6[b]	14.7[b]	2.4[b]
	AC 383	0.0	0.8[b]	7.0[b]	13.3[b]	15.1[b]	2.4[b]
	AC 407	0.0	0.8[b]	7.0[b]	13.3[b]	15.3[b]	25[b]
	AC 450	0.0	0.8[b]	6.0[b]	12.1[b]	14.3[b]	2.4[b]
	AC 492	0.0	0.8[b]	6.5[b]	13.0[b]	14.8[b]	2.4[b]
	AC 536	0.0	0.8[b]	6.0[b]	12.2[b]	14.4[b]	2.4[b]
	AC 564	0.0	0.8[b]	7.0[b]	12.4[b]	14.7[b]	2.5[b]
	AC 568	0.0	0.8[b]	6.8[b]	12.0[b]	14.4[b]	2.4[b]
	AC 581	0.0	0.8[b]	6.0[b]	12.0[b]	14.3[b]	2.3[b]
	AC 640	0.0	0.8[b]	6.4[b]	12.5[b]	14.7[b]	2.5[b]
	AC 644	0.0	0.8[b]	6.3[b]	12.5[b]	14.9[b]	2.4[b]
	AC 680	0.0	0.7[b]	6.0[b]	12.5[b]	14.9[b]	2.5[b]
	AC 742	0.0	0.8[b]	6.0[b]	12.3[b]	14.6[b]	2.5[b]
	AC 810	0.0	0.8[b]	7.2[b]	13.0[b]	15.0[b]	2.6[b]
	AC 845	0.0	0.8[b]	6.0[b]	12.0[b]	14.3[b]	2.4[b]
	AC 857	0.0	0.8[b]	6.3[b]	12.8[b]	14.7[b]	2.3[b]
	AC 972	0.0	0.8[b]	5.6[b]	12.6[b]	14.7[b]	2.4[b]
	AC 220	0.0	0.8[b]	6.0[b]	12.5[b]	14.6[b]	2.5[b]
	AC 420	0.0	0.8[b]	6.5[b]	13.0[b]	15.1[b]	2.5[b]
	AC 456	0.0	0.8[b]	7.0[b]	13.0[b]	15.2[b]	2.4[b]
	AC 487	0.0	0.8[b]	7.0[b]	13.2[b]	15.3[b]	2.5[b]
	AC 496	0.0	0.7[b]	6.4[b]	12.3[b]	14.7[b]	2.4[b]
	AC523	0.0	0.8[b]	6.0[b]	12.4[b]	14.6[b]	2.4[b]
	AC 639	0.0	0.7[b]	6.0[b]	12.3[b]	14.4[b]	2.4[b]
	AC 673	0.0	0.8[b]	7.2[b]	13.0[b]	15.1[b]	2.5[b]
	AC 718	0.0	0.8[b]	6.5[b]	12.5[b]	14.8[b]	2.5[b]

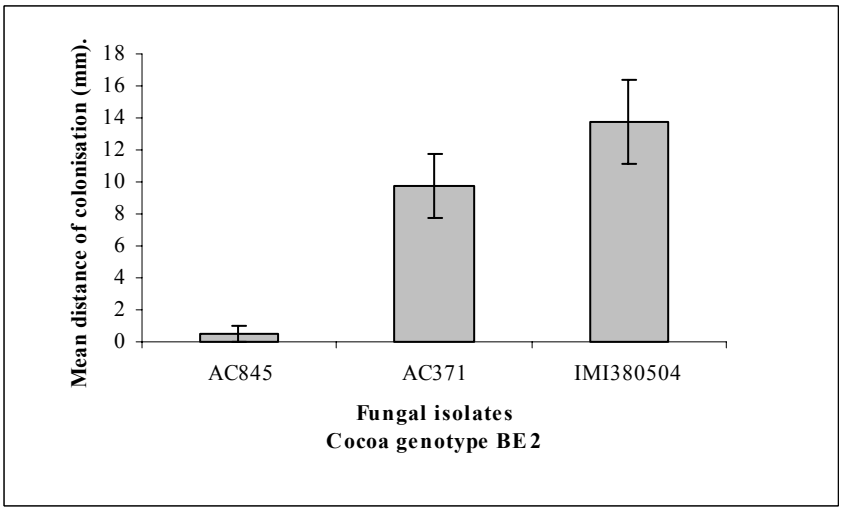
Appendix 2. Fungal colonisation* of stems of cocoa germplasm lines in the laboratory.

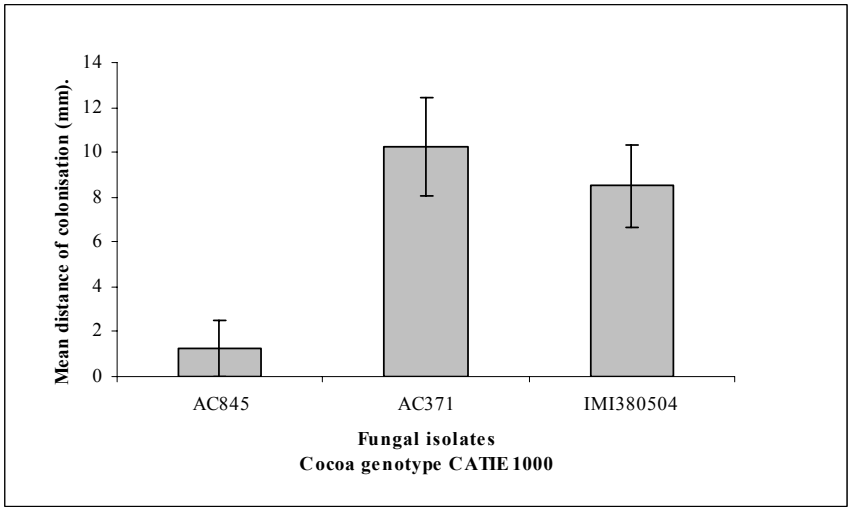


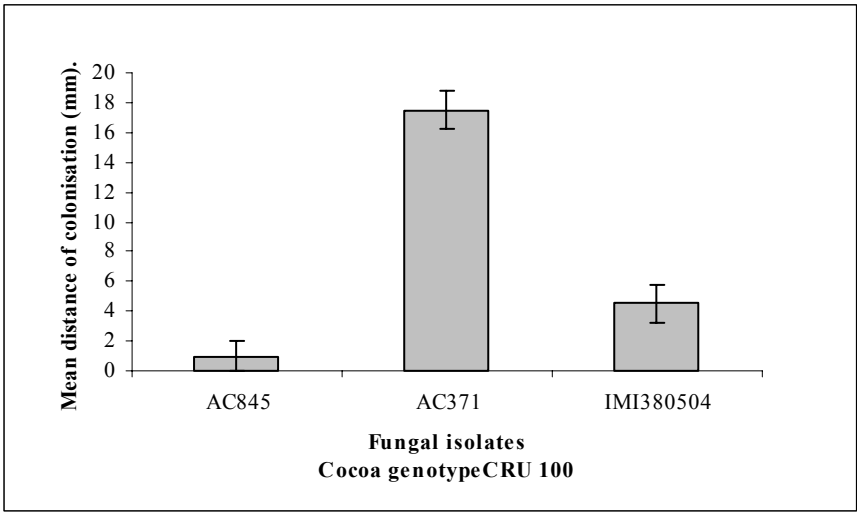


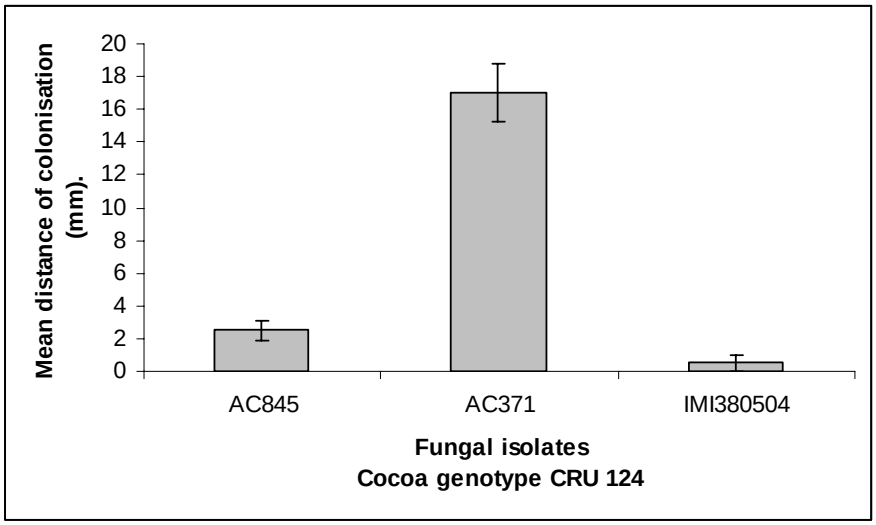


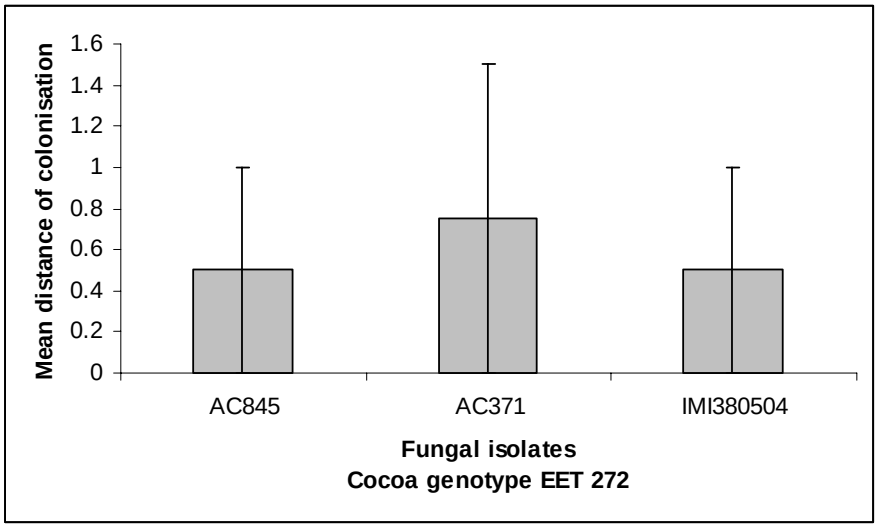

Mean distance of colonisation (mm).
18
16
14
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype Amelonado

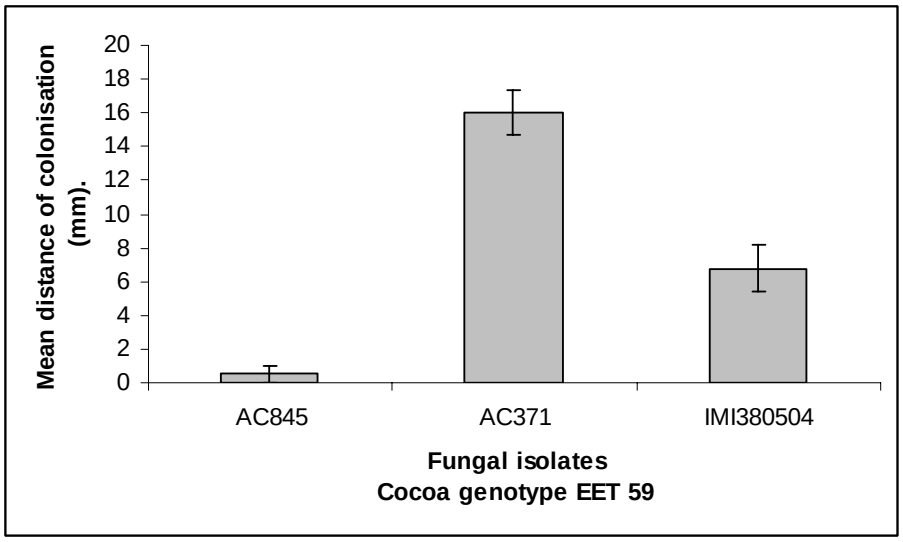

Mean distance of colonisation (mm).
18
16
14
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype BE2

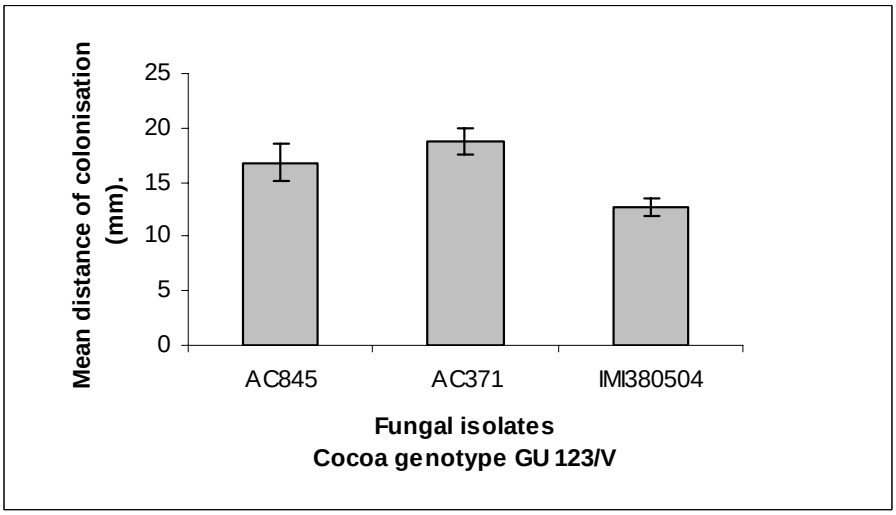

Mean distance of colonisation (mm).
14
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype CATIE1000

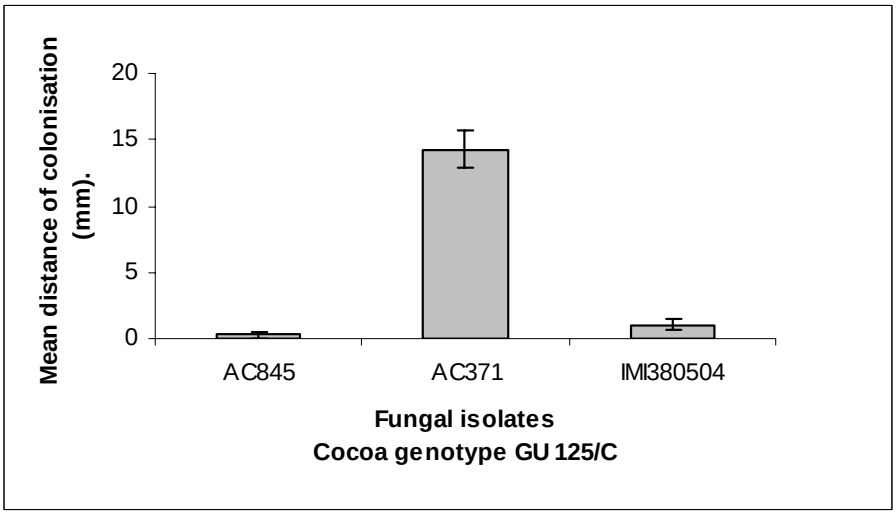

Mean distance of colonisation (mm).
20
18
16
14
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotypeCRU 100

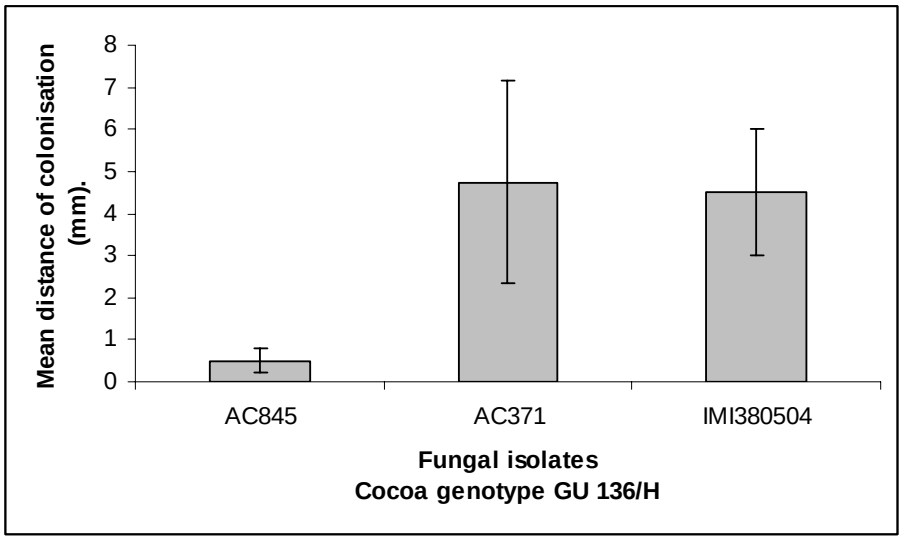

Mean distance of colonisation (mm).
20
18
16
14
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype CRU 124

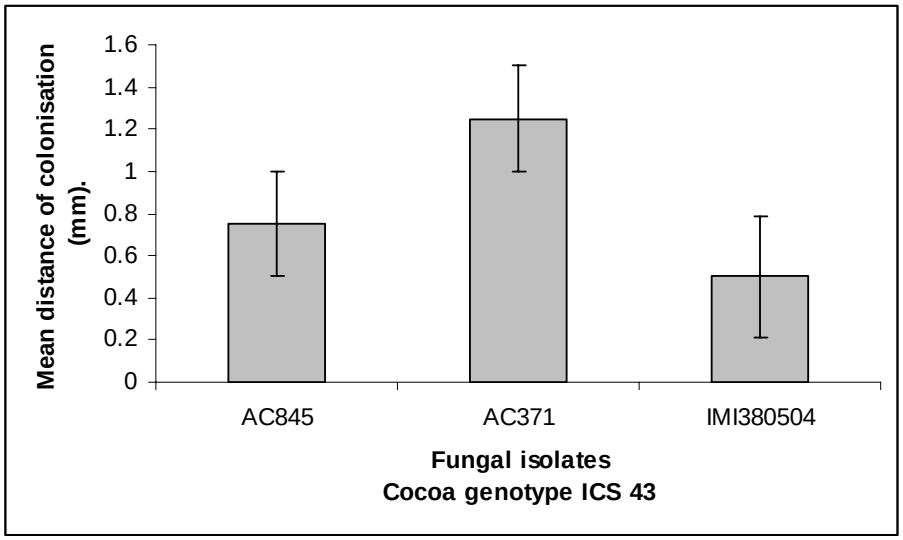

Mean distance of colonisation
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype EET 272

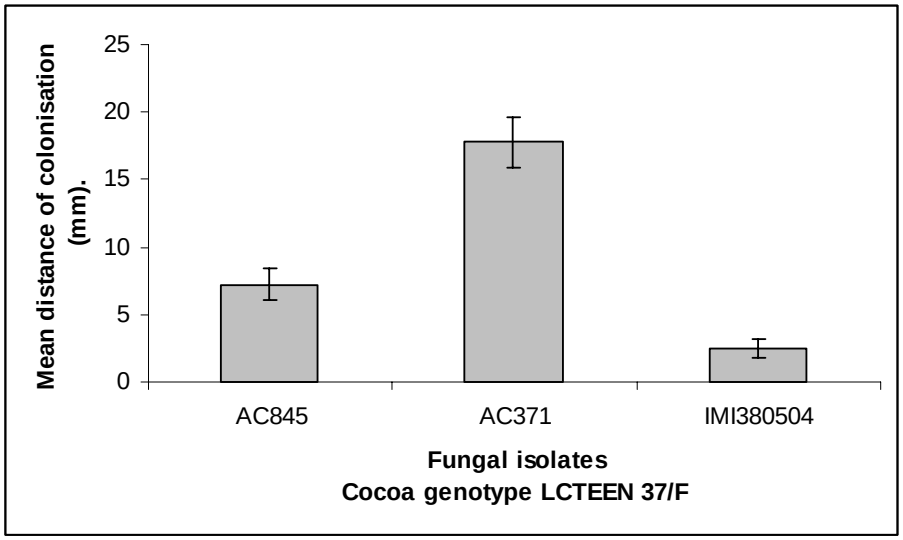

Mean distance of colonisation (mm).
20
18
16
14
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype EET 59

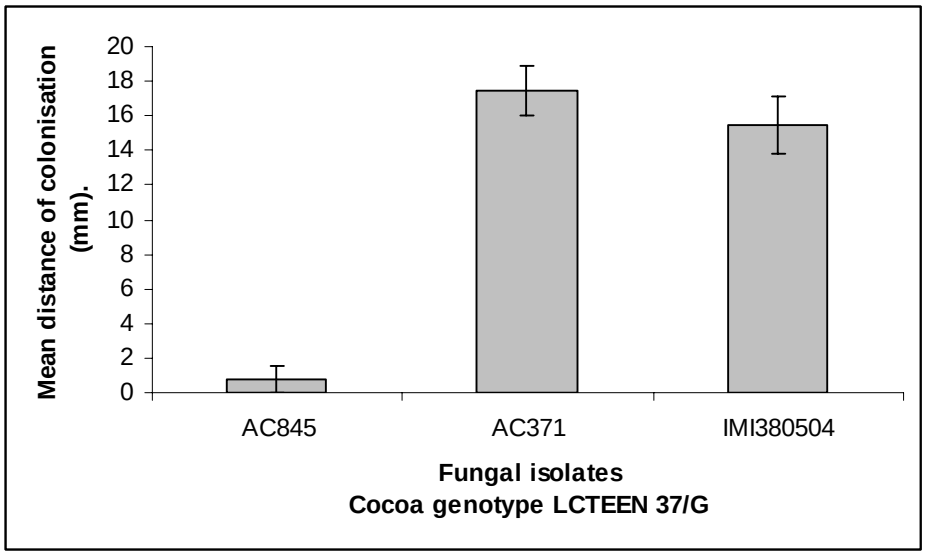

Mean distance of colonisation (mm).
25
20
15
10
5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype GU 123/V

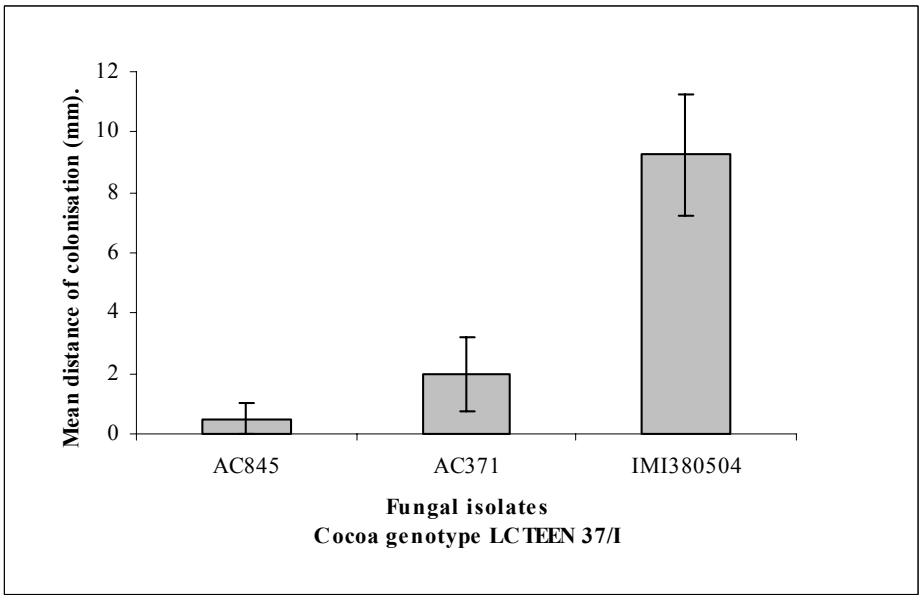

Mean distance of colonisation (mm).
20
15
10
5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype GU 125/C

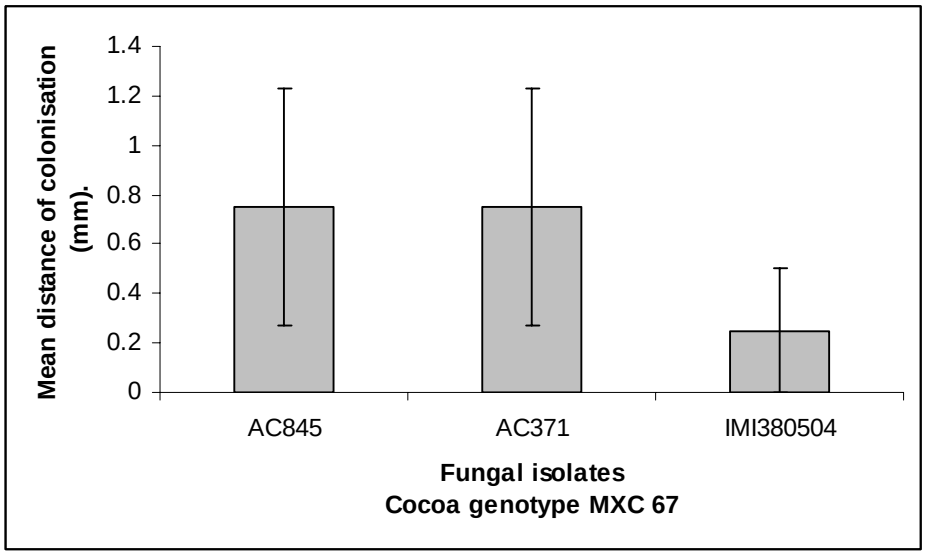

Mean distance of colonisation (mm).
8
7
6
5
4
3
2
1
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype GU 136/H

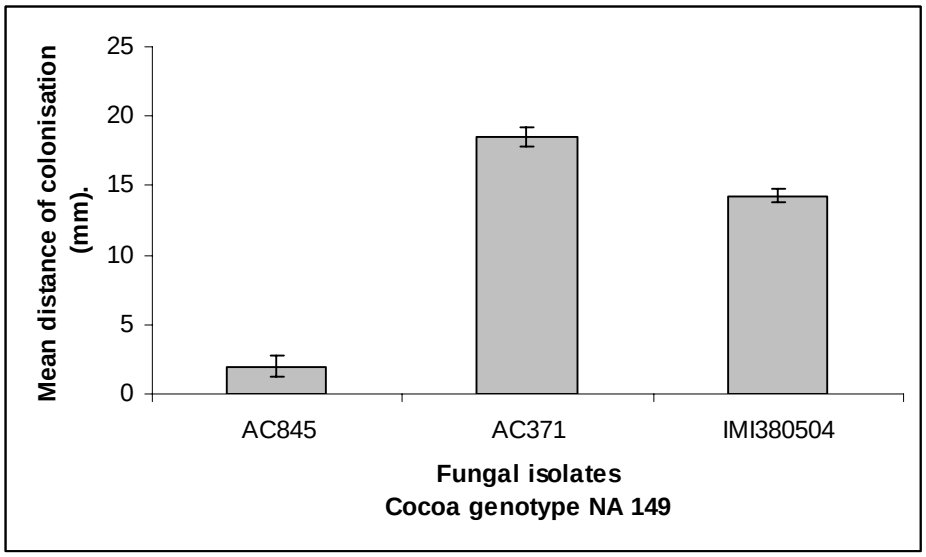

Mean distance of colonisation (mm).
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype ICS 43

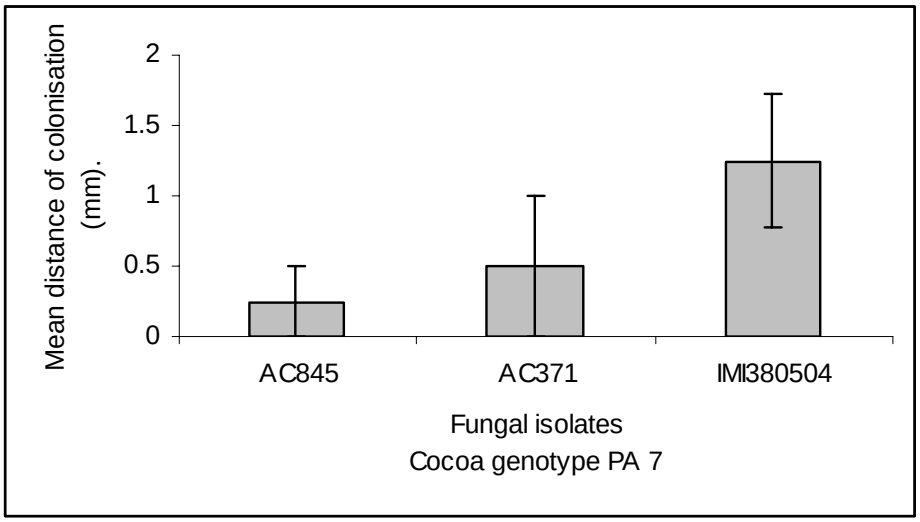

Mean distance of colonisation (mm).
25
20
15
10
5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype LCTEEN 37/F

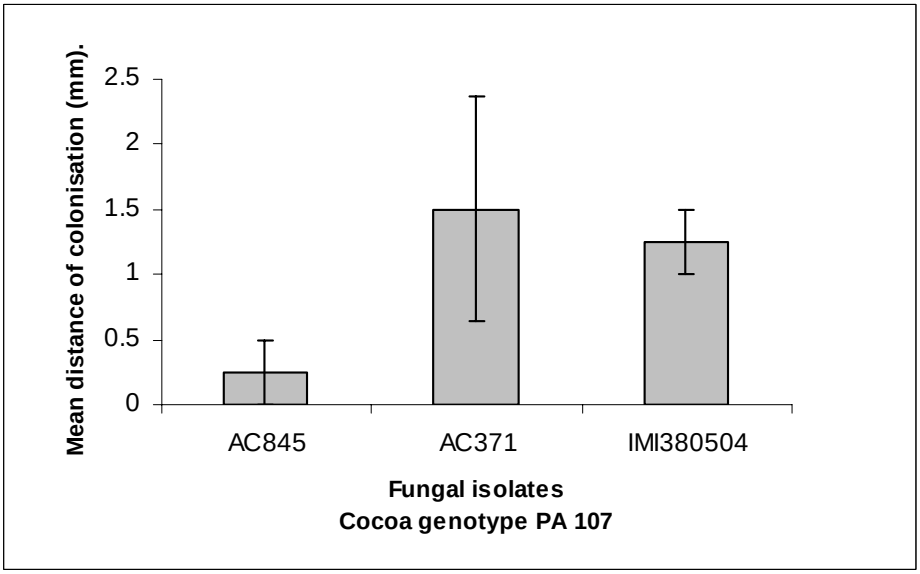

Mean distance of colonisation (mm).
20
18
16
14
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype LCTEEN 37/G

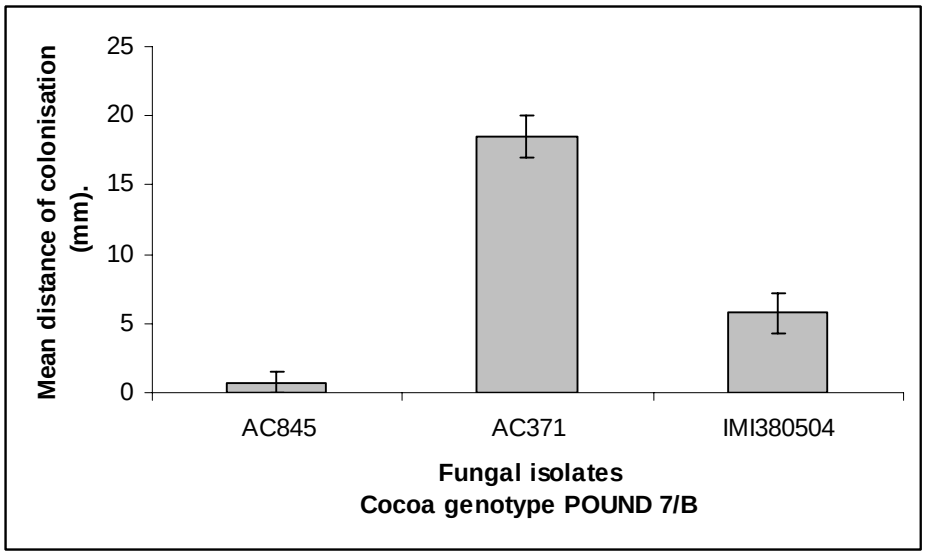

Mean distance of colonisation (mm).
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype LCTEEN 37/I

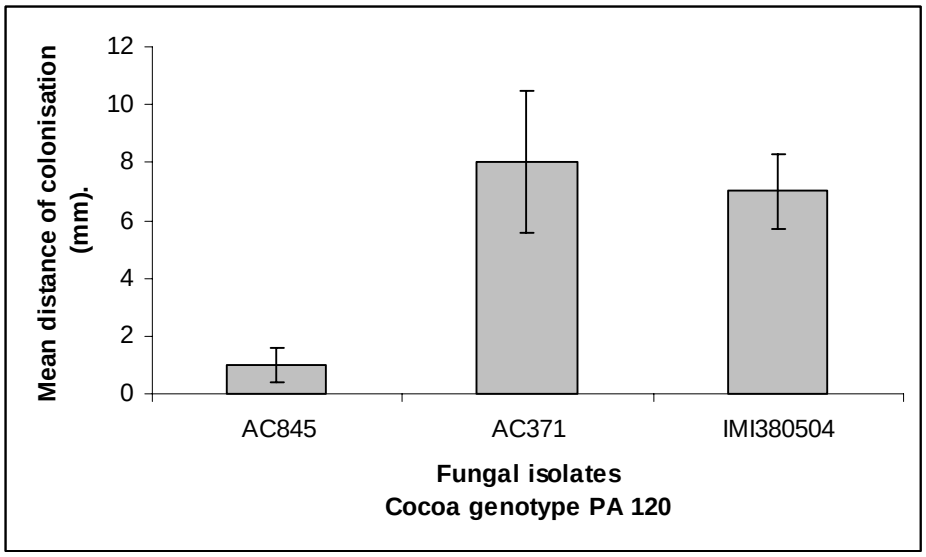

Mean distance of colonisation (mm).
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype MXC 67

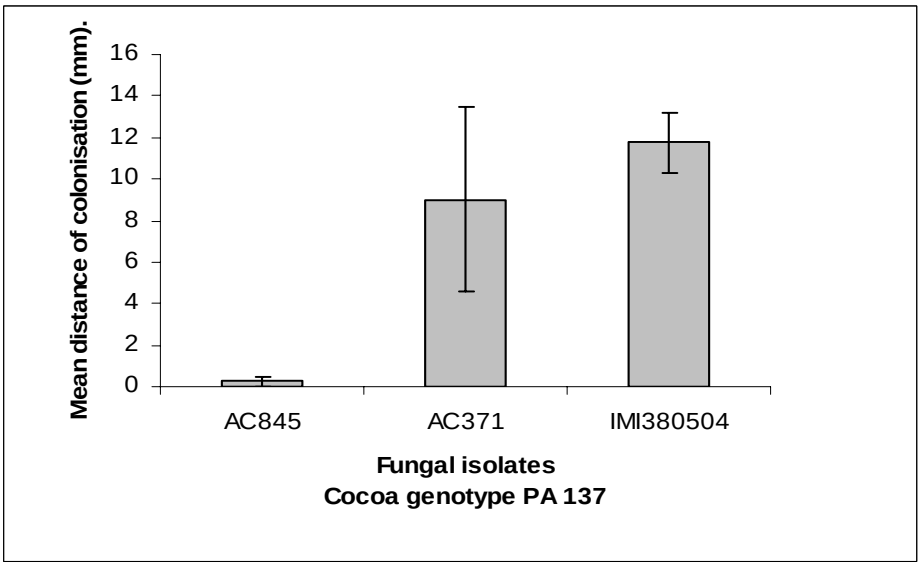

Mean distance of colonisation (mm).
25
20
15
10
5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype NA 149

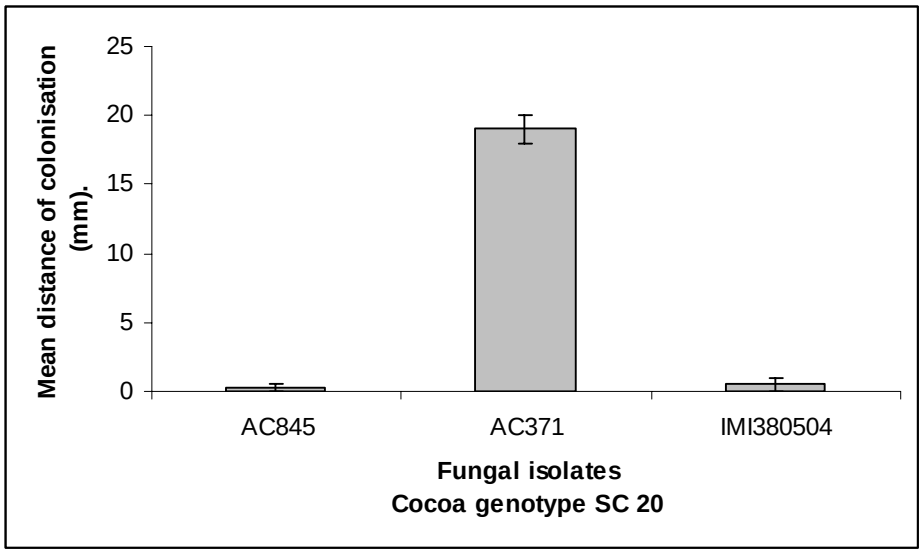

Mean distance of colonisation (mm).
2
1.5
1
0.5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype PA 7

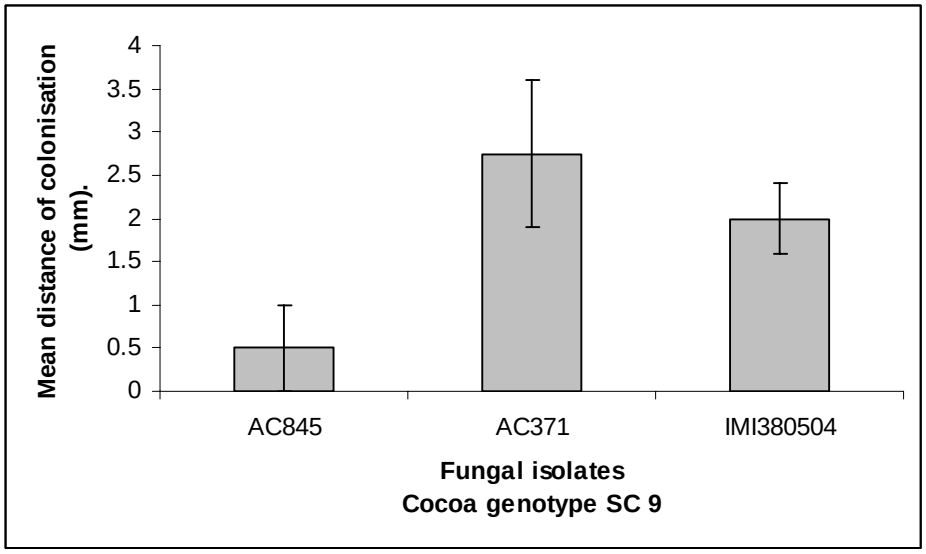

Mean distance of colonisation (mm).
2.5
2
1.5
1
0.5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype PA 107

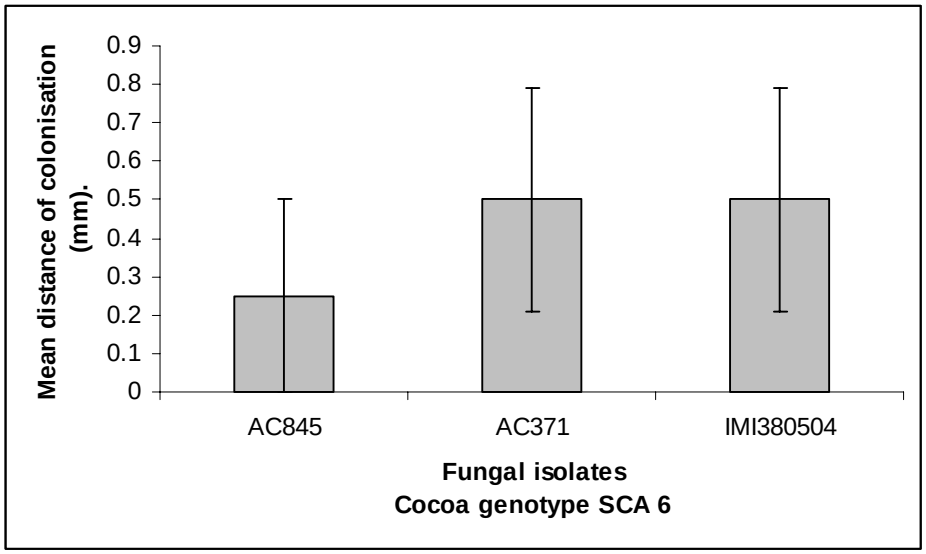

Mean distance of colonisation (mm).
25
20
15
10
5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype POUND 7/B

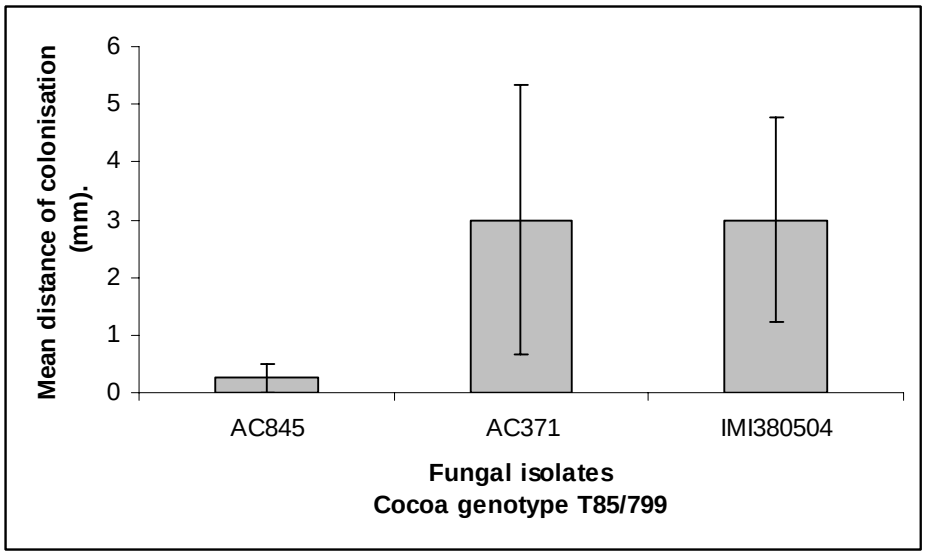

Mean distance of colonisation (mm).
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype PA 120

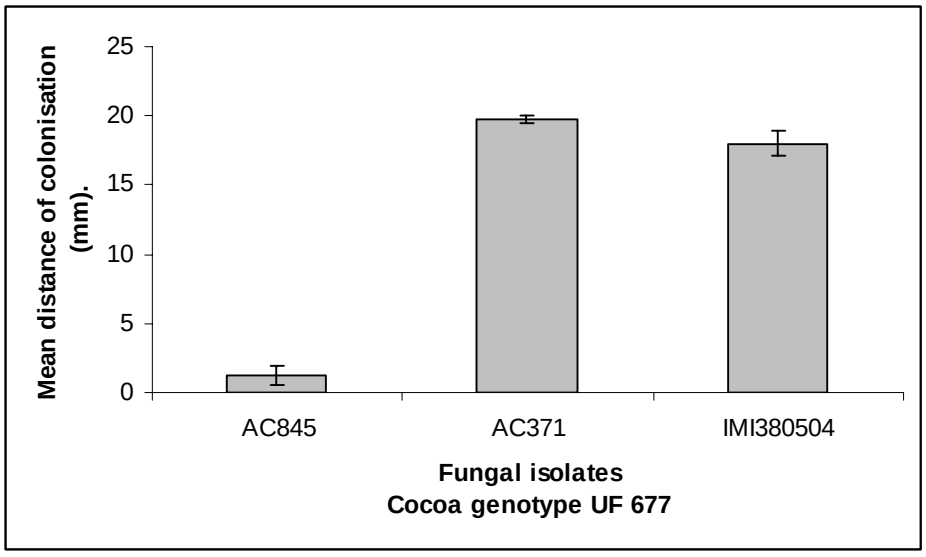

Mean distance of colonisation (mm).
16
14
12
10
8
6
4
2
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype PA 137


Mean distance of colonisation (mm).
25
20
15
10
5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype SC 20


Mean distance of colonisation (mm).
4
3.5
3
2.5
2
1.5
1
0.5
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype SC 9


Mean distance of colonisation (mm).
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
AC845
AC371
IMI380504
Fungal isolates
Cocoa genotype SCA 6





*Differences between isolate effects shown on the vertical axis was exaggerated by default in excel.

What can I give Him

Poor as I am;

If I were a shepherd,

I would give Him a lamb.

If I were a wise man,

I would do my part-

But what can I give Him?

I will give Him my heart.

- Christina Rossetti

To God be the Glory