Fungal biomass distribution in witches' broom disease of cocoa

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by

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Corrections

For *herbicide*, page 5, paragraph 2, lines 2 and 5, read *fungicide*. Also, for *herbicides*, page 5, paragraph 4, line 2, read *fungicides*.

For *Table 2.10*, page 18, paragraph 2, line 1, read *Table 2.1*.

Page 24, paragraph 1, line 4 , the sentence: "Likewise, ratios between glucose, mannose, glucosamine, and uronic acid in various combinations probably would not have allowed the calculation of the relative concentrations of vegetative and sporulating biomass", should read: "Ratios between glucose, mannose, glucosamine, and uronic acid in various combinations may not have allowed the calculation of the relative concentrations of vegetative and sporulating biomass".

For *absorbtion*, page 30, paragraph 1, line 6, read *absorption*.

Page 69, paragraph 2, line 4, the sentence: "Thus there appeared to be great variety in lipid content between a large number of fungal species, and this was true even for very closely related species", should read: "Thus there appeared to be great variety in lipid content between a number of fungal and *bacillus* species, and this was true even for very closely related species".

For *Entomorpha*, page 70, paragraph 2, line 1, read *Entomophthora*.

Page 83, paragraph 1, line 3, the sentence: "However, the concentration of zeatin riboside, the presumed inactive bound form of zeatin, was found to differ significantly in broom tissues in relation to uninfected tissues", should read: "However, the concentration of zeatin riboside was found to differ significantly in broom tissues in relation to uninfected tissues".

"To change the situation it's going to need a lot more than talking, we need policies being made at the level of western governments. A policy decision has to be made to give indigenous peoples a real place, their true place in the international concert of nations"

Rigoberta Menchú,

speaking

to a

New York Times' journalist

For my mum, dad,

Maureen, gran,

and

grandad.

Acknowledgements

Firstly, special thanks must go to my supervisors Sue Isaac, Hamish Collin, and Keith Hardwick. I also wish to thank my 'unofficial' supervisor George Britton, from the Department of Biochemistry. Without George's help, lab space and explanations, much of this project would have been an awful lot harder. I would also like to thank Professor Rees, also from the Department of Biochemistry, who never raised an eyebrow at the often absurd hours I put in while working on the GC. The technical staff in the Department of Biochemistry, and in the Department of Environmental and Evolutionary Biology also deserve much appreciation for their help. I am also indebted to Case Veltkamp and Jim Smith for their help with the electron microscope. I also wish to thank my project sponsors the Biscuit, Cake, Chocolate & Confectionery Alliance, UK. Very special thanks must go to Sue Edwards, who was always a great source of advice.

Abbreviations

ANOVA	analysis of variance
°C	degrees centigrade
cm	centimetre
Dept.	department
dia	diameter
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
et al.	*et alii*
FID	flame ionisation detector
Fig.	figure
g	gram
GC	gas chromatograph
HPLC	high performance liquid chromatography
hr	hour
Ltd.	public limited company
min	minute
ml	millilitre
PEG	polyethylene glycol
pers. comm.	personal communication
sec	second
ng	nanogram
M:C	mannan chitin ratio
mg	milligram
mm	millimetre
MS	Mass spectrometer
rpm	revolutions per minute
RI	refractive index detector
SD	standard deviation
SDS	sodium dodecyl sulphate

Abbreviations continued

SEM	scanning electron microscope
TEM	transmission electron microscope
µg	microgram
UK	United Kingdom
µl	microlitre
USA	United States of America
v/v	volume/volume percentage
w/v	weight/volume percentage
w/w	weight/ weight percentage

Summary

The basidiomycete fungus *Crinipellis perniciosa* (Stahel) Singer is the causative agent of witches' broom disease of cocoa (*Theobroma cacao*). Witches' broom disease is endemic to large areas of the Americas and is a major constraining factor on cocoa production in such areas.

The disease cycle includes a primary monokaryotic biotrophic phase and a secondary dikaryotic saprotrophic phase. In order to study the early stages of infection the possibility of quantifying the relative concentrations of the primary and secondary phases using changes in the ratio of mannan to chitin was investigated. Both chitin and mannan are fungal cell wall polymers.

Separate gas chromatographic based mannan and chitin assays were developed. The new chitin estimation procedure was significantly more sensitive than the commonly used colorimetric based methods and was also selective for chitin rather than a range of similar components. The new method required only about 200 μg of material whilst the previous and colorimetric based procedures required approximately 25 mg. Thus, the new procedure was sufficiently sensitive to detect extremely low levels of infection in clearly defined plant regions, such as meristems. The mannan assay was not significantly more sensitive than the previous assays, but produced cleaner samples, and thus interpretation of results was simplified and mass spectrometer maintenance reduced.

To capitalise on the new chitin and mannan assays, the work was extended to encompass neutral and amino sugars. No useful biomarkers or ratios between components were discovered. Ratios between sugars may have been capable of apportioning the relative concentration of primary and secondary phase *C. perniciosa* in infected cocoa.

All extractable lipids were also evaluated as potential biomarkers, and the lipid profiles assessed for potentially useful ratios. No useful lipid ratios were discovered, but ergosterol was found to be a useful biomarker.

The newly developed chitin assay was used to study *C. perniciosa* infection in green and brown brooms. The assay was also used to investigate the distribution of *C. perniciosa* in sections taken throughout the length of an entire broom. The results indicated *C. perniciosa* was highly localised at the broom base and at the growing points.

The use of random amplified polymorphic DNA polymerase chain reaction as a guide to pathogenicity was also evaluated. The method was adapted for use with *C. perniciosa* at the Institut Für Genbiologische Forschung, Berlin. The method indicated the presence of fungal plasmids in the primary phase of *C. perniciosa* but not in the secondary.

Contents

Chapter 1

Introduction

Cocoa is one of the world's most important crops. Many millions of people in the third world rely on it for their survival. It also forms the main hard currency export of many nations in west Africa, south America and the Caribbean.

The main product of cocoa, cocoa butter, is an extremely valuable lubricant. However, chocolate is the most recognisable product of cocoa. Chocolate and confectionery are major industries world-wide, employing many tens of thousands of people. A glance at the Financial Times will show just how important the confectionery industry is, with household names like Cadbury, Rowntree Mackintosh, and Mars featuring prominently. Nestlé, who own Rowntree Mackintosh, is even a constituent of the FT-SE Eurotrack 100, and as such is one of Europe's largest companies.

Fifty Kit Kats are eaten every second, 17,000 Smarties are consumed per minute in Britain alone, while more than 2,000,000,000 Quality Street sweets are produced per year. But Britain doesn't just consume chocolate, it also exports it in massive quantities. For example, Rolo is exported to more than 50 countries, and children can be seen in the sands of Saudi Arabia munching Cadbury's milk chocolate, as an extra source of calcium and protein.

Most chocolate production and consumption occurs in Europe and north America. While all cocoa production occurs in the tropics, with the Caribbean, south America and west Africa producing the most. In spite of its importance, cocoa production is relatively small in volume terms by commodity standards, only 1.5 million tonnes are produced annually. However, whole economies are supported by cocoa production. Banana republic may apply to Guatemala, but cocoa 'democracy' must surely apply to Trinidad & Tobago.

However, all is not well. Cocoa prices have been falling, impoverishing many hundreds of thousands of peasant families across the tropics. And, international agreements aimed at stabilising world prices are faltering (Financial Times, 1993). Long term, the cocoa price will rise, but an even more important spectre is appearing; the continuing spread of witches' broom disease.

Witches' broom was first described by Went in 1904. The disease is caused by the basidiomycete fungus *Crinipellis perniciosa* (Stahel) Singer. It was first identified in Surinam as *Marasmius perniciosus*, by Stahel (1915), and subsequently transferred to the genus *Crinipellis* by Singer (1942). The classic symptom of the disease is the so called 'broom' (Fig. 1.1). Brooms consist of swollen and distorted branches, flowers, and shoots. These brooms grow from infected vegetative buds and occasionally from flower cushions. The rampant growth frequently takes on the appearance of the witches' broom described in numerous fairy tales.

The spores of *Crinipellis perniciosa* infect actively growing meristematic tissues and the symptoms of the disease to a large extent reflect the nature and stage of development of these tissues (Wheeler, 1985). The classic symptoms, the brooms, tend to be produced from vegetative buds and flower cushions. These so called 'green brooms' are associated with the primary phase of the fungus. The primary phase of *C. perniciosa* is believed to be monokaryotic, and therefore lacks clamp connections (Pegus, 1972; Evans, 1980). This has been disputed by Delgado & Cook (1976). The fungal hyphae are relatively wide (5-20 μm), frequently swollen and flexuous (Pegus, 1972; Evans, 1980), and grow intercellularly in parenchymatous tissue (Calle, 1978; Calle *et al.*, 1982). The primary phase is the biotrophic form of the fungus.

Eventually the green broom dies and forms a brown broom. The brown broom is associated with the secondary phase of *C. perniciosa*. The secondary phase mycelia are relatively thin (1.5-3 μm), dikaryotic and therefore possess clamp connections. They grow both intra- and intercellularly throughout all tissues (Pegus, 1972). The secondary phase mycelia are saprotrophic and give rise to fruiting bodies known as basidiomes, which subsequently produce basidiospores.

The fungus is endemic to the forests of the Amazon basin. It grows on wild cocoa, related species of *Theobroma* and *Herrania*, Lianas, *Solanum*, and also on material from the understorey and litter (Thorold, 1975; Evans, 1978; Bastos & Evans, 1985; Hedger, 1985). Just

as the Incas believed cocoa was a gift from the gods, cocoa cultivation in a tropical environment is a gift to *Crinipellis.*

With the advent of commercial cocoa cultivation in the early part of this century, *Crinipellis* began to spread steadily across south America and the Caribbean. Witches' broom disease has severely curtailed cocoa production in Bolivia, Colombia, Ecuador, Grenada, Guyana, Peru, Trinidad & Tobago, and Venezuela (Periera *et al.*, 1990). The disease therefore blights millions of lives across the Americas. Within the last three years the disease has been reported in Bahia, Brazil (Periera *et al.*, 1990). Bahia is one of the most important cocoa growing regions in the world, representing 85% of Brazil's cocoa production.

In 1978 the Brazilians were so concerned about the threat from *Crinipellis* that a phytosanitary belt was set-up encompassing strategically placed entrances to Bahia state. Other measures were also introduced, such as educating the populace to look out for the symptoms of witches' broom and the dangers of bringing in contaminated materials. Sometime in 1989 the phytosanitary belt was breached (Periera *et al.*, 1990). The Brazilians are now trying to contain the spread of *C. perniciosa* in an economically vital zone.

All the more disturbing is the fact that the disease almost certainly did not spread to Bahia naturally. There is a powerful argument that man introduced *C. perniciosa* to Bahia (Periera *et al.*, 1990). Whether by chance or malice, the disease is threatening to become a continent wide pandemic. If the disease spreads to other cocoa growing countries outside the Americas, then millions more lives will be blighted (Wood & Lass, 1985). Chocolate production and a major chunk of Britain's industry would also suffer.

There has been much research aimed at controlling witches' broom disease. One of the most interesting investigations was performed by Bastos *et al.* (1986). The authors were investigating the host range of *C. perniciosa* and found a hyperparasite capable of killing it. The fungus was identified as *Cladobotryum amazonense* sp. nov. They found that the fungus physically prevented basidiospore release and produced a toxin capable of plasmolysing

basidiospores. And when applied to susceptible plant surfaces the toxin prevented infection by *C. perniciosa*. The toxin was subsequently identified by Bastos *et al.* (1986) as a heat stable polypeptide.

More conventional herbicide research has been performed by McQuilken *et al.* (1988). The authors investigated the effectiveness of two triazole herbicides at preventing the symptoms of witches' broom disease. Hexaconazole gave good control when used as a spray drench while triadimenol worked best as a pre-inoculation soil drench. Great differences in susceptibilities to the herbicides between different *C. perniciosa* isolates were also found, thus undermining the potential effectiveness of a control program based on these herbicides.

To date, the most exhaustive study into the control of witches' broom disease has been performed under the auspices of the International Witches' Broom Project (IWBP). This programme was carried out at 21 centres in Europe, Latin America, and the United States, with the aim of reducing the devastation caused by witches' broom. The seven year project was funded by the chocolate manufacturers of Europe and north America. Many international aid agencies and the governments of 6 cocoa producing countries also contributed.

The project aimed to find the most effective and practical means of controlling witches' broom. Control measures using herbicides and phytosanitation were evaluated. Phytosanitation, the removal and destruction of all potentially infected material, proved to be the most practical means of controlling witches' broom. But, the project organisers stressed that ultimately the most effective means of control would probably be through a resistance breeding programme.

Because of the low cocoa price, at present it is not possible to economically control witches' broom disease in large areas of the Americas. The only potentially effective means of control require either large inputs of man-made chemicals, or a great deal of extra labour. Therefore, large sums of money must be invested to increase the yield by reducing the effects of witches'

broom disease. Any system requiring large inputs of capital will not be viable in a cocoa plantation. Thus, other means of controlling witches' broom are required.

A great deal of research has been conducted on *C. perniciosa* and witches' broom disease of cocoa. But still, many of the fundamental aspects of the disease are not known. It is difficult to envisage attempts at controlling say, rust, without first understanding in reasonable detail the basic dynamics of the disease. This approach was acceptable when scientific knowledge and technologies were limited. This is no longer the case. In the past, many plant fungal pathogens were controlled to some degree before the disease was intimately understood. However, this solution is no longer acceptable or necessary. The techniques are available, or can be adapted for use in cocoa, so that the dark areas in our knowledge of *C. perniciosa* and its interactions with cocoa can be enlightened.

Although the outline life cycle is believed to be understood, very little is known about other fundamental aspects of the disease. For example, little is known about the morphological, physiological, and biochemical interactions between cocoa and *C. perniciosa*. Recently, work describing some fundamental aspects of such interactions has been published (Isaac, Hardwick, & Collin, 1992).

One of the main deficiencies in our knowledge of host pathogen interactions is the mechanics of fungal development within the host. For example, disease symptoms vary enormously, but it is not known how these relate to fungal biomass. Nor is it known how the relative proportions of primary and secondary phase mycelia affect disease symptoms. There is also no information concerning the effect on disease symptoms of dikaryotisation timing, or on the effects of relative activities of the primary and secondary phases.

If these questions could be answered, or at least clarified, then the chances of designing effective control strategies for the disease would be greatly improved. If the details of the life cycle were understood then the effectivness of phytosanitation may be improved. If it was known how the balance of primary and secondary phases affect disease symptoms then it

may be possible to rationally design cheap herbicides which favourably shift the balance between the two phases. And if the physiology of the disease was understood it may be possible to improve the screening procedures to enhance natural cocoa resistance to *C. perniciosa*.

Attempts have been made to identify resistant cultivars of cocoa. Little natural resistance has been found, and this has all too frequently 'broken-down' in the cocoa plantation environment. The need for more resistant varieties or ecotypes is great, especially as witches' broom is now spreading rapidly throughout Bahia. If the basics of the interactions between *C. perniciosa* and cocoa are more deeply understood then the chances of success in the search for resistant varieties will be greatly enhanced.

The research described in this thesis was financed by the Biscuit, Cake, Chocolate & Confectionery Alliance, UK, and was concerned with developing biochemical methods for quantifying fungal material in host tissues, and relating these to severity of disease symptoms. Ideally, such techniques would allow the separate quantification of both the primary and secondary phases of the fungus within the host.

The advantages of a biochemical technique are numerous. Manual screening using microscopy is time consuming. In addition, when low levels of fungal invasion occur, it may be extremely difficult if not impossible to detect a few fungal hyphae in sections taken from, for example, a leaf or stem. Although fungal specific stains make life somewhat easier. Also, quantitative data often cannot be obtained using a microscopy based technique. However, recent advances in stereology are beginning to change the situation (Edwards, 1993). Hicks & Newell (1984) outlined two further advantages of a biochemical based technique; samples may often be stored for longer periods, and also less subjectivity in identification is required by the investigator.

The key advantages of a biochemical technique over a microscopy based technique is the speed, accuracy, and sensitivity that quantitative data can be obtained from a single or small group of biological samples.

In the past, information acquired about *C. perniciosa* has been gained through epidemiological and microscopy based studies. As a result, new techniques of investigation were needed in order to further our understanding of the disease. Therefore, the aim of this work was to develop new biochemical methods to identify and quantify the different growth forms of *C. perniciosa* in infected cocoa material. It would then be possible to investigate the effects of the relative proportions of the primary and secondary phases on the development of disease symptoms.

This thesis is divided into 8 chapters. Chapter 2 deals with the present state of knowledge of witches' broom. It also concerns the culturing techniques developed for the primary phase of the fungus. Chapter 3 describes the development of biochemical study techniques using the fungal biomarkers chitin and mannan, while Chapter 4 takes this work further by exploring the potential of other neutral and amino sugars. The fifth chapter looks at the potential of lipid biomarkers, most notably ergosterol. Chapter 6 contains the investigation of witches' broom disease using the newly developed and highly sensitive chitin assay. The seventh chapter concerns preliminary molecular biology work performed at the Institut für Genbiologische Forschung, Berlin. The final chapter is an overall summary, and contains the future directions of research that I feel are most appropriate to take.

Fig. 1.1. A representative witches' broom.

Chapter 2

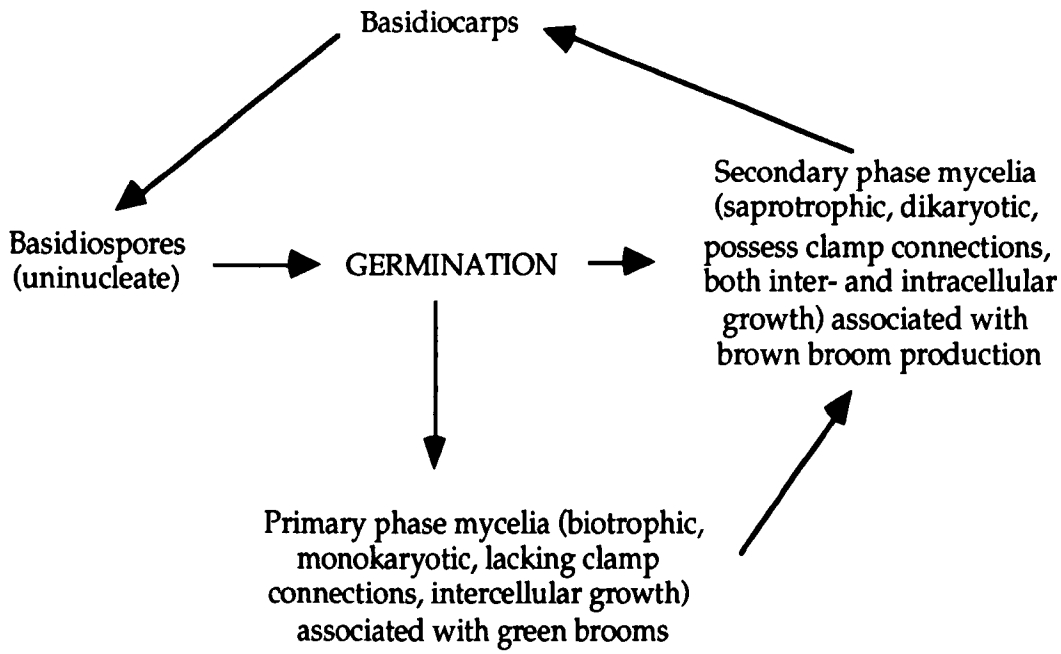
The fungus

2.1.1a Introduction

The fungal pathogen *C. perniciosa* has a hemibiotrophic life-style. The primary phase of the fungal life cycle is initiated when *C. perniciosa* basidiospores land on buds, flower cushions, or other actively growing regions of cocoa plants (Cronshaw & Evans, 1978). The spores germinate on the plant surface and the germ tubes quickly penetrate the host. Initially, the primary phase of the fungus grows intercellularly and biotrophically (McGeary & Wheeler, 1988). During the primary phase the fungus is monokaryotic and lacks clamp connections (Pegus, 1972; Evans, 1980). The hyphae are relatively wide (5-20 μm) and are frequently swollen and flexuous (Pegus, 1972). After infection, the host cocoa begins to produce distorted growth resembling the typical witches' broom, hence the name of the disease. Such distorted growth is produced because of loss of apical dominance and the production of multiple meristems. Regions of distorted growth are termed 'green brooms' (Stahel, 1915). It is not known how or why the host responds in this manner. No disturbance has been found in plant hormone levels, although significantly raised levels of zeatin riboside have been found in infected cocoa tissue (Orchard *et al.*, 1993).

The green brooms frequently 'grow-through', i.e. the plant returns to a more normal pattern of growth, although the original, distorted material remains and frequently produces normal looking cocoa tissue. (Hardwick pers. comm., Dept. Environmental and Evolutionary Biology, University of Liverpool, UK). Often the green broom dies and turns into a 'brown broom' (Stahel, 1915). Brown brooms are associated with the secondary phase of *C. perniciosa*. The secondary phase is dikaryotic and possesses clamp connections (Pegus, 1972). The hyphae are thinner (1.5-3 μm) than primary phase hyphae and grow both intra- and intercellularly, and throughout all cocoa tissues (Pegus, 1972). The secondary phase grows saprotrophically and produces fruiting bodies known as basidiomes. The trigger which induces the switch from the primary to the secondary phase of growth is not known, nor is the manner by which the fungus causes host tissue death.

The *C. perniciosa* life-cycle as proposed by Evans (1980) is shown below.



Recent work by Brownlee *et al.* (1990) provided further information concerning the gross differences in morphology between the primary and secondary phase mycelia. The swollen and flexuous mycelia, regarded as a characteristic of the primary phase (Pegus, 1972), have been produced by cultures of *C. perniciosa* in the presence of host tissue extracts (Brownlee *et al.*, 1990). More significantly, the degree of expression of these characteristics was related to the concentration of the cocoa extracts. There was no simple switch between the two characteristic morphologies of the primary and secondary phases of growth as would be expected from Evans' (1980) dual mycelial theory (Brownlee *et al.*, 1990). Indeed the results obtained by Brownlee *et al.* (1990) were more consistent with a concentration dependent alteration of cell growth processes by an inhibitor. Further work by Brownlee *et al.* (1990b) discovered that this inhibitor was polymeric procyanidin (condensed tannin). The authors suggested that polymeric procyanidin may play a direct role in cocoa resistance to witches' broom disease.

The secondary phase of the fungus is relatively easy to grow in culture, and will grow on such complete media as V8 juice agar (Aragundi, 1982). The primary phase of the fungus is

less easy to produce in culture for longer than a few days (Evans, 1980). Hedger *et al.*, (1987) succeeded in maintaining primary phase *C. perniciosa* in culture for up to 8-9 days. Orchard (pers. comm., Dept. Environmental and Evolutionary Biology, University of Liverpool, UK) succeeded in growing primary phase *C. perniciosa* mycelia in culture for 10-12 days. Ten or 12 days probably would not have been sufficient for samples of the fungus to produce enough biomass for the experiments outlined in the rest of this thesis. Therefore the first investigation in this chapter was designed to see if it was possible to produce primary and secondary phase *C. perniciosa* mycelia in sufficient quantities for the experimental work outlined in the rest of the thesis. The Manaus F isolate was used in all the investigations detailed in this thesis because it is one of the few which fruit in culture, and was kindly supplied by B. E. J. Wheeler (Dept. Pure and Applied Biology, Imperial College, UK).

The key distinguishing difference between the primary and secondary phases of *C. perniciosa* is the respective karyotype (Pegus, 1972). The primary phase is monokaryotic, while the secondary is dikaryotic (Pegus, 1972; Evans, 1980). The primary phase of *C. perniciosa* does not form clamp connections, whilst the secondary phase does (Pegus, 1972; Evans, 1980). Hedger *et al.* (1987) distinguished between the primary and secondary phases of *C. perniciosa* on the basis of presence or absence of clamp connections when studying pathogenic variability of the fungus. Therefore, the presence of clamp connections was used to distinguish between samples of primary and secondary phase *C. perniciosa*. The second investigation in this chapter was designed to determine the length of time the primary phase could be maintained in culture before switching to the secondary phase. The third investigation was designed to produce growth curves for the primary and secondary phases of *C. perniciosa* in order to determine the optimum harvesting time for the primary and secondary phases of *C. perniciosa*. The optimum harvesting time would be when individual samples had accumulated 20-30 mg of biomass as the initial work in Chapter 3 required this quantity.

2.1.1b Methods

In accordance with COSHH regulations, all the work in this thesis was conducted with the aid of appropriate safety measures.

Production of basidiomes

Basidiomes and hence basidiospores for the production of primary phase mycelia were obtained using the method of B. E. J. Wheeler (pers. comm., Dept. Pure and Applied Biology, Imperial College, UK). Aliquots (30 ml) of 1.5 % (w/v) water agar were poured into 250 ml conical flasks. Prior to autoclaving, the flasks were sealed with absorbent cotton wool and covered with aluminium foil. After autoclaving the agar was allowed to set. Sterile cocoa twigs, previously autoclaved at 121 °C for 1 hr, were placed vertically onto the agar surface. Plugs (10 mm dia) of stock secondary phase *C. perniciosa* (Manaus F strain), grown as described below, were placed at the base of the sterile twigs, bridging the gap between the twig and agar surface. The conical flasks were re-sealed and incubated at 23 °C, in constant light, for 3-12 months.

Production of primary phase mycelia

Aliquots (5 ml) of potato dextrose liquid, made up as recommended by the manufacturer, were placed in 30 ml glass universal bottles. The bottles were autoclaved, and a film of Vaseline spread over the underside of the lids, which were then surface sterilised with ethanol. Basidiomes were removed from the cocoa twigs by their stipes using sterile forceps. The basidiomes were stuck onto the Vaseline with the gills pointing away from the lids. The lids were then replaced on the vials and incubated as static cultures at 23 °C in constant light.

After 18 days the resulting fungal hyphae were harvested for light and electron microscopy. Samples of hyphae were also placed onto V8 juice agar and adjacent to actively growing *C. perniciosa* Manaus F, to check for anastomoses. If, after 2 weeks the colony edges had visibly merged with no signs of incompatibility then anastomosis was considered to have occurred. Anastomosis indicated that the colonies were compatible and therefore of the same species.

Squash preparations of the hyphae were produced for light microscopy. The squash preparations were produced by gently squashing a segment of a representative colony (2 mm dia) between a glass slide and cover slip. The hyphae were viewed and photographed under a microscope with a calibrated eyepiece. Samples of hyphae were also fixed for transmission electron microscopy (TEM), see below.

Maintenance of primary phase mycelia in culture

Three replicate sub-samples were taken from eight samples of primary phase mycelia every 7 days. Squash preparations for each sample were then produced and viewed under a light microscope, as described earlier. For each replicate, all hyphae in 20 frames of view were inspected for the presence of clamp connections. The primary phase of the fungus was considered to have switched to the secondary form if any clamp connections were observed. The cultures were sub-cultured into fresh potato dextrose liquid after 28 days and the observations continued.

Production of a primary phase growth curve

Spore derived cultures of primary phase *C. perniciosa* were produced as described earlier. The cultures were harvested after 12, 18, and 25 days. The resulting hyphae were then freeze-dried, weighed, and a growth curve produced. The exclusive presence of primary phase hyphae in the spore derived cultures was confirmed as described earlier, using light microscopy. The exclusive presence of *C. perniciosa* was confirmed using anastomosis, also described earlier.

Maintenance of stock secondary phase cultures

The Manaus F secondary phase culture, supplied by B. E. J. Wheeler, was sub-cultured onto V8 juice agar, consisting of 10 % V8 juice (v/v) and 1.5 % agar (w/v). The agar was autoclaved for 15 min at 121 °C and poured into petri dishes. Portions of the original *C. perniciosa* culture were then transferred to the V8 juice agar and incubated at 25 °C in constant light. The stock *C. perniciosa* colonies were sub-cultured onto fresh V8 juice agar every 2 weeks.

Production of secondary phase mycelia for biochemical analysis

Secondary phase *C. perniciosa* mycelia were also grown in potato dextrose liquid, as described below, in order to study the secondary phase under the same conditions as those used to produce the primary phase. Aliquots (50 ml) of potato dextrose liquid, prepared as recommended by the manufacturer, were poured into 250 ml conical flasks. The flasks were then sealed with absorbent cotton wool overlaid with aluminium foil. The flasks were then autoclaved for 15 min at 121 °C.

A stock inoculum of secondary phase *C. perniciosa* was produced as follows. Conical flasks (250 ml) containing 50 ml of glass beads (3-6 mm dia), and 100 ml of potato dextrose liquid, were sealed and autoclaved as described in the previous paragraph. One hundred plugs (6 mm dia) of 14 day old secondary phase *C. perniciosa* Manaus F grown on V8 juice agar were added to the glass bead containing flasks. The flasks were then violently agitated by hand for 3-4 min to completely macerate the agar plugs. Aliquots (5 ml) of this stock inoculum were then added to the potato dextrose liquid containing flasks. The flasks were then sealed and incubated at 23 °C on a rotating platform, swirling at 50 revolutions per min, in constant light.

After 18 days, the resulting fungal biomass was harvested for light and electron microscopy. Samples of the hyphae were also placed onto V8 juice agar adjacent to actively growing colonies of *C. perniciosa* to check for anastomosis. Squash preparations, produced as described earlier, of the fungal samples were produced for light microscopy. The hyphae were viewed and photographed using a microscope with a calibrated eyepiece. Samples of hyphae were also fixed for TEM as described below.

Production of a secondary phase growth curve

Cultures of secondary phase *C. perniciosa* grown in potato dextrose liquid were produced as described earlier. The cultures were harvested after 11, 21, 27, 35 and 41 days. The resulting hyphae were then freeze-dried, weighed, and a growth curve produced. The presence of secondary phase hyphae in the cultures was confirmed using light microscopy, as described

earlier. The exclusive presence of *C. perniciosa* was confirmed in the cultures using anastomosis, as described earlier.

Preparation for TEM

The samples were initially fixed in 5 % glutaraldehyde (w/v) in 0.1 M sodium cacodylate buffer (pH 7.2) for 24 hr at 4 °C. The samples were then washed twice in 0.1 M sodium cacodylate buffer (pH 7.2) over 30 min. The samples were then post-fixed in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 hr at room temperature, then washed in two changes of the same buffer. The samples were then dehydrated through an alcohol gradient and infiltrated with Spurr's (1969) resin. The alcohol gradient consisted of 30, 50, 70, and 90 % ethanol, plus three changes of absolute ethanol. The samples were soaked in the 30 % solution for 15 min and the others for 30 min.

The samples were infiltrated with resin as follows: the ethanol was poured off, leaving enough to cover the sample. To the ethanol, an equal volume of resin was added and the mixture left for 30 min. Another volume of resin was then added and the mixture allowed to stand for a further 30 min. The mixture was then poured off and a new volume of resin added and left for 30 min before another volume of resin was added, left for 30 min, and poured off again. Once again, another volume of resin was added. The resin was then left for 12 hr. The resin was then poured off, another volume added, and then allowed to cure for 16 hr in an oven at 60 °C.

The resin and sample block was then sectioned on a Reichert-Jung Ultracut E ultra microtome to produce sections with a thickness of 90-100 nm using a 45° glass knife. The sections were then placed on a copper grid (300 mesh) and stained for 25 min with a saturated solution of uranyl acetate in 50 % ethanol. After staining, the sections were washed in 50 % ethanol and then in distilled water, and finally dried with filter paper. The sections were then post-stained with Reynolds' (1963) lead citrate for 9 min. The sections were then washed in 0.02 M NaOH and then in distilled water before drying with filter paper. The sections were viewed under a Zeiss EM10CR transmission electron microscope with an accelerating voltage of 60 KV.

2.1.1c Results and Discussion

A representative cocoa twig used for basidiome production is shown in Fig. 2.1. Basidiome production generally began within 3 months, and lasted a further 3-6 months. Basidiomes tended to be produced in groups of 2 or 3 rather than singly. Total basidiome production ranged from 15-20 for a typical cocoa twig.

Fig. 2.2 and 2.3 show representative longitudinal sections of primary phase *C. perniciosa* when viewed under the TEM. Fig. 2.4 and 2.5 show representative primary phase *C. perniciosa* hyphae viewed under the light microscope. The majority of the hyphae were in the size range 2-3 μm in diameter and all septa were totally lacking in clamp connections. The hyphae were relatively unbranched in comparison with the secondary phase mycelia and appeared full of cytoplasm and organelles.

Fig. 2.6 and 2.7 show representative longitudinal sections of secondary phase *C. perniciosa* hyphae when viewed under the TEM. Fig. 2.7 demonstrates clearly the presence of clamp connections. Fig. 2.8 and 2.9 show representative secondary phase hyphae when viewed under a light microscope. The majority of the hyphae were approximately 2-3 μm in diameter. Many septa possessed clamp connections and the hyphae were relatively highly branched in comparison with the primary phase. The hyphal compartments were occasionally devoid or partially devoid of cytoplasm and organelles.

Hyphae produced directly from spores did not bear clamp connections and were therefore considered to have formed primary phase mycelia. Hyphae isolated from the plate cultures and grown in potato dextrose liquid under the same conditions as the hyphae produced from spores did bear clamp connections. Other workers have also produced primary phase *C. perniciosa* directly from spores, but the cultures switched to the secondary, clamp connection bearing phase within 2 days (Evans, 1980). Hedger *et al.* (1987) using the same media components as this work, potato dextrose, but as an agar not a liquid culture, maintained *C. perniciosa* in culture for 8-9 days. Likewise, Orchard (pers. comm.) maintained the primary phase of the fungus in culture for 10-12 days on potato dextrose agar.

More than 2,000 views of the hyphae grown from spores or from plate cultures using both light and electron microscopy were examined. Clamp connections were not observed in the spore derived or primary phase cultures but were observed in the plate derived or secondary phase cultures. The results presented here demonstrated that both the primary and secondary phase mycelia lacked gross differences in morphology. Pegus (1972) and Evans (1980) showed that the primary phase mycelia when in cocoa tissue were flexuous, swollen, and relatively wider (5-20 μm) than the secondary phase mycelia (1.5-3 μm). The results presented here did not correspond to those observations. However, Brownlee *et al.* (1990; 1990b) found that the thick, swollen, and flexuous mycelia were only produced in the presence of host cocoa extracts or more specifically polymeric procyanidin, in the absence of this component the typical, thin, secondary phase mycelia were produced. The data outlined here was obtained in cultures of potato dextrose liquid and therefore lacked host cocoa tissue extracts. Thus, this data may add further weight to the work of Brownlee *et al.* (1990; 1990b) that the differences in gross morphology between the primary and secondary phase mycelia are a response by the fungus to host cocoa tissue, or extracts thereof. In the absence of such extracts there were no gross differences in morphology between the primary and secondary phases of growth, thus the major difference between the two phases was the nuclear state. The difference between the primary and secondary phase mycelia in this work was the absence of clamp connections in the primary phase, and thus the monokaryotic nature of the mycelia, and the presence of clamp connections in the secondary phase, and thus the dikaryotic nature of the mycelia.

Maintenance of the primary phase in culture

The results (Table 2.10) clearly demonstrated that the primary phase cultures remained in the primary phase for at least 28 days, the time of sub-culturing. No clamp connections were seen in any replicate treatment for the first 28 days. However, within 7 days of sub-culturing into fresh potato dextrose liquid media, a few clamp connections were formed, and within 14 days of sub-culturing, clamp connections were clearly visible in all replicate treatments. Within 18-25 days sufficient primary phase biomass per sample was generally produced to allow the experiments detailed in the remainder of this thesis to proceed. Thus, primary

phase hyphae could be harvested within 18-25 days for any of the required experimental work. Evans (1980) managed to produce and keep *C. perniciosa* as a primary phase culture for up to 2 days. No other workers were able to maintain *C. perniciosa* as a primary phase culture for longer than the 10-12 days achieved by Orchard (pers. comm.). The ability to maintain *C. perniciosa* as a primary phase culture was probably due to the combination of the *C. perniciosa* strain and the media used for the experiments. Evans (1980) maintained the primary phase mycelia of an unknown strain of *C. perniciosa* on water agar but they rapidly (24-48 hr) produced secondary phase hyphae. Orchard (pers. comm.) maintained the primary phase of *C. perniciosa* 555 for 10-12 days on potato dextrose agar. Whereas this work was performed with *C. perniciosa* Manaus F and using potato dextrose liquid. Hedger *et al.* (1987) also using potato dextrose agar, maintained the primary phase in culture for 8-9 days. The difference between the semi-solid potato dextrose agar, and the potato dextrose liquid, was unlikely to have alone produced the differences observed here, they were almost certainly due to the different *C. perniciosa* strains used in the experiments. Other workers (Hedger *et al.*, 1987; Wheeler & Mepstead, 1988) have found major differences in pathogenicity and compatibility between different *C. perniciosa* isolates, it is likely that the ability to be maintained as a primary phase culture also differs significantly between strains. Manaus F exhibits several features unusual for *C. perniciosa*, most notably the ability to produce basidiomes in axenic culture. To this, another unusual characteristic of Manaus F has been discovered, the ability to be maintained as a primary phase mycelial culture. By further manipulating the culture conditions it is likely that the primary phase mycelia could be maintained in culture for an even greater length of time.

Primary and secondary phase growth curves

The results for the primary phase growth curve (Fig. 2.10) clearly demonstrated the required quantity of biomass (20-30 mg) was produced within 18-25 days. Therefore, the optimum harvesting time was after 18 days. By day 18, sufficient biomass had accumulated and therefore a point of diminishing returns was reached after this time. The spore derived primary phase cultures reached stationary phase after about 25 days.

The results for the secondary phase growth curve (Fig. 2.11) demonstrated the required quantity of biomass was produced within 15-21 days. Therefore, the optimum harvesting time was after approximately 18 days. Harvesting after 18 days would also provide another level of consistency with the primary phase mycelia grown under identical conditions.

The secondary phase cultures grew actively for longer than the primary phase, forming stationary phase mycelia after 35-41 days. The relatively longer growth phase of the secondary phase mycelium was almost certainly due to the greater absolute starting level of nutrients in the conical flasks. The conical flasks contained approximately 10 times the quantity of nutrients as the universal bottles used for growing the primary phase mycelia, although the concentration was identical. Thus, the secondary phase cultures grew for longer and attained a greater stationary phase biomass.

No other workers have been able to produce a growth curve for cultures of primary phase *C. perniciosa*, because others have only been able to maintain a primary phase *C. perniciosa* culture for a relatively short period of time. Hedger *et al.* (1987) and Orchard (pers. comm.) were unable to produce primary phase *C. perniciosa* growth curves because the hyphae began producing secondary phase hyphae after only 8-9 and 10-12 days respectively. Thus, the hyphae had switched life-styles before growth curves could be produced. The use of the culture conditions described here, plus the Manaus F strain of *C. perniciosa*, allowed for the first time, the production of a primary phase *C. perniciosa* growth curve.

2.1.1d Conclusions

Spore derived cultures of primary phase *C. perniciosa* were maintained for more than 28 days in the culture system described. After sub-culturing the 28 day old cultures, the primary phase switched to the clamp connection forming secondary phase. The work also demonstrated the required 20-30 mg per sample of primary and secondary phase *C. perniciosa* biomass required for experimental purposes was attained after about 18 days. Therefore, samples of primary and secondary phase *C. perniciosa* cultures were harvested after 18 days for the experiments detailed in the rest of this thesis.

Fig. 2.1. A representative 3 month old cocoa twig used for basidiocarp production.

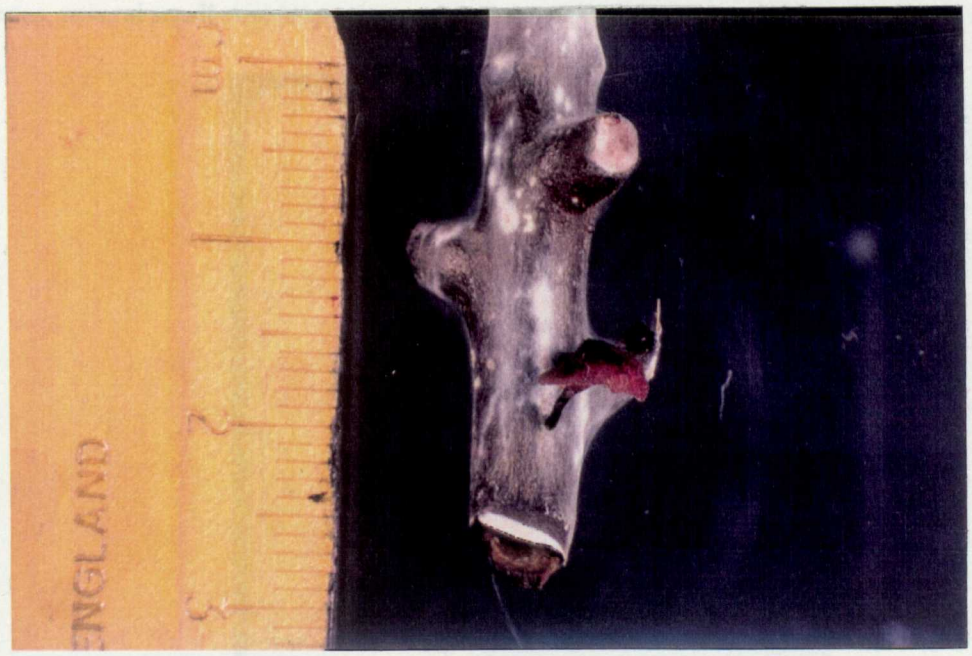


Fig. 2.2. Transmission electron micrograph of a longitudinal section through part of a hypha in the primary phase of *C. perniciosa*, showing mitochondria (M), endoplasmic reticulum membranes (ER), osmophilic material (OS), and the cytoplasm densely packed with ribosomes, several small vesicles and vacuoles (V) are also present. Scale bar equals 0.5 μm.

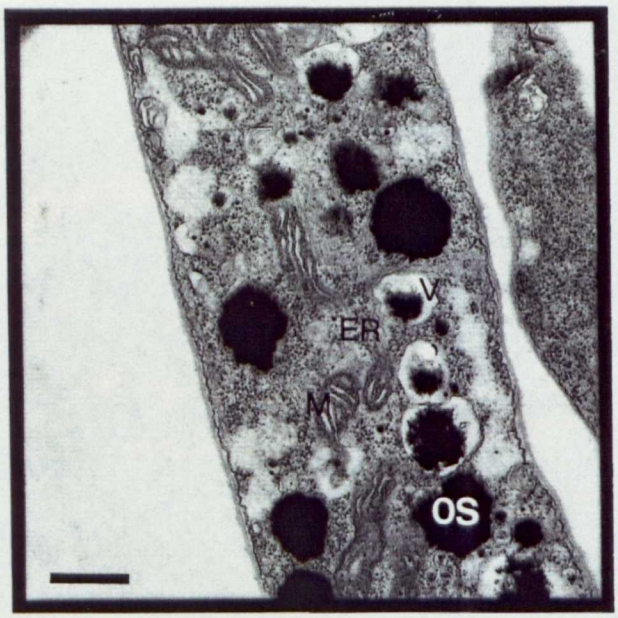


Fig. 2.3. Transmission electron micrograph of a longitudinal section through part of a hypha in the primary phase of *C. perniciosa*, showing septal pore (sp) apparatus. The septal pore swellings (SW) contain electron opaque material adjacent to the cross wall (SE). The plasmalemma (P) is continuous between adjacent cells. Continuity of the endoplasmic reticulum (ER) and cap (SC) is evident. Within the septal pore black bodies are evident, these may be plugs. Scale bar equals 0.25 μm.

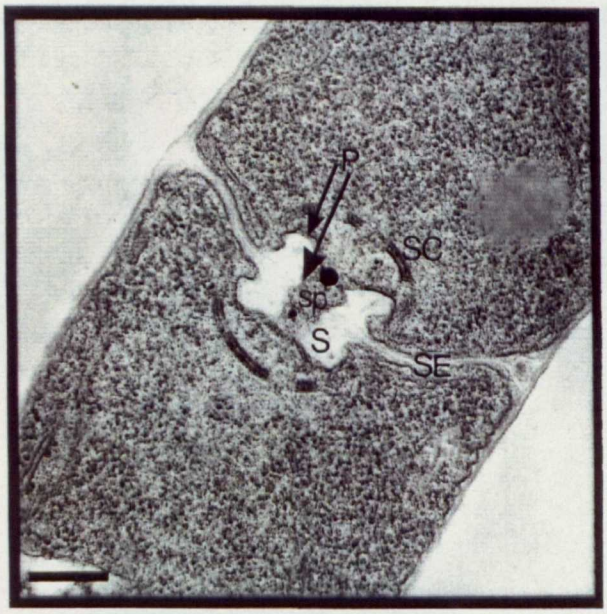


Fig.2.4. Phase contrast micrograph of the primary phase of *C. perniciosa*. Note the lack of clamp connections (⟶) between septal cells. Scale bar equals 5 µm.

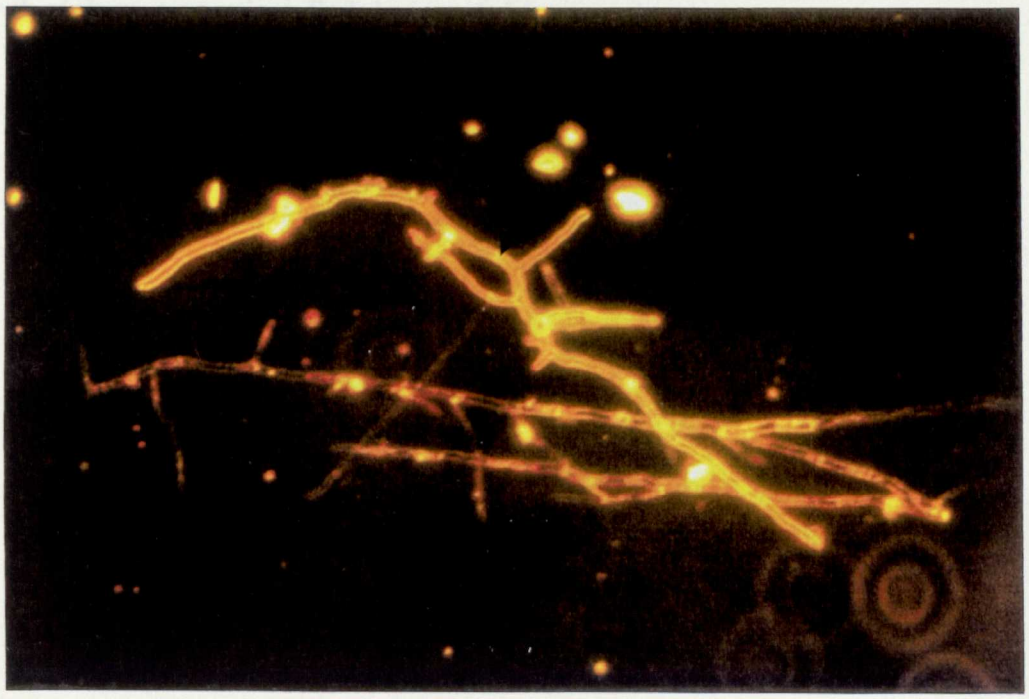


Fig.2.5. Phase contrast micrograph of the primary phase of *C. perniciosa*. Note the lack of clamp connections (⟶) between septal cells. Scale bar equals 2 µm.

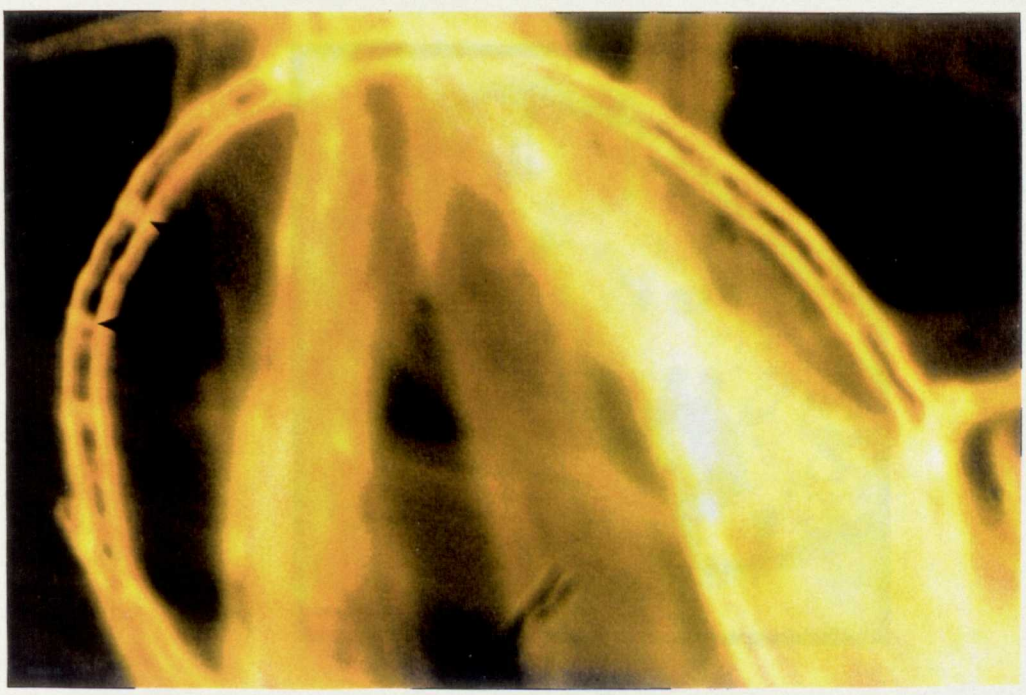


Fig. 2.6. Transmission electron micrograph of a longitudinal section through part of a hypha in the secondary phase of *C. perniciosa*. Two septal compartments are visible (S1 and S2); a septal pore (SP) allows movement of solutes between the two, a clamp connection (CL) is in the process of developing. Many vesicles (V) are present and are known to be involved in the growth of clamp connections. Scale bar equals 0.5 μm.

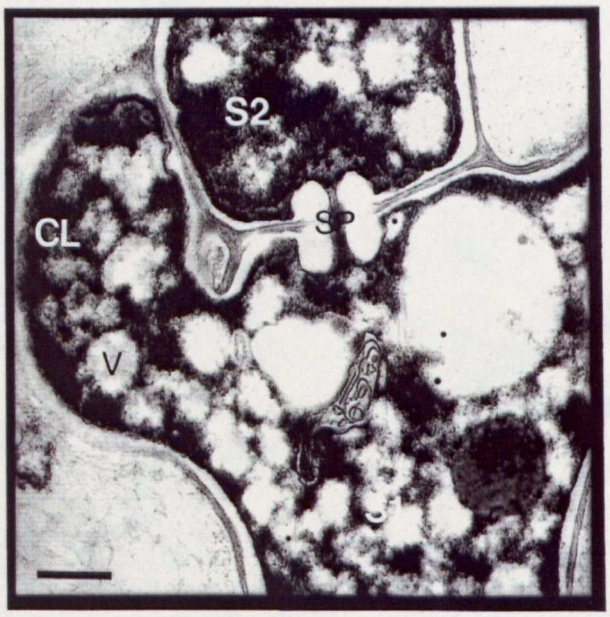


Fig. 2.7. Transmission electron micrograph of a longitudinal section through part of a hypha in the secondary phase of *C. perniciosa*. Again two septal compartments are visible (S1 and S2); a septal pore (SP) is also visible. A clamp connection (CL) has developed in the second septal compartment (S2) behind the hyphal front (HP). Scale bar equals 0.5 μm.

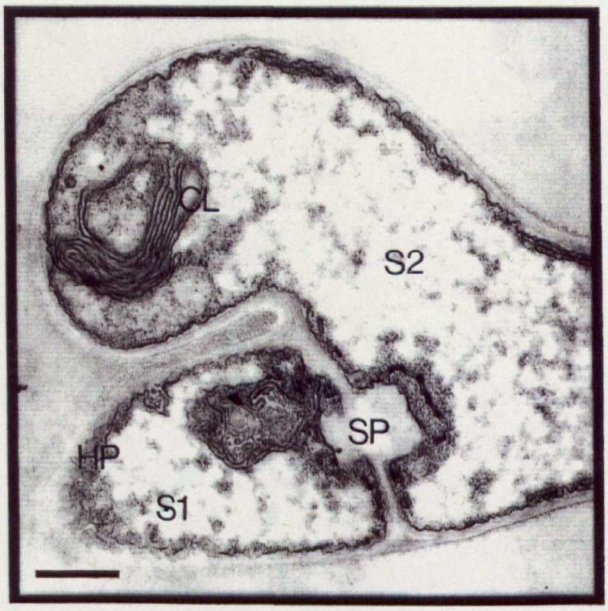


Fig 2.8. Phase contrast micrograph of the secondary phase of *C. perniciosa*. Note the presence of clamp connections (⟶) between septal cells. Scale bar equals 2 µm.

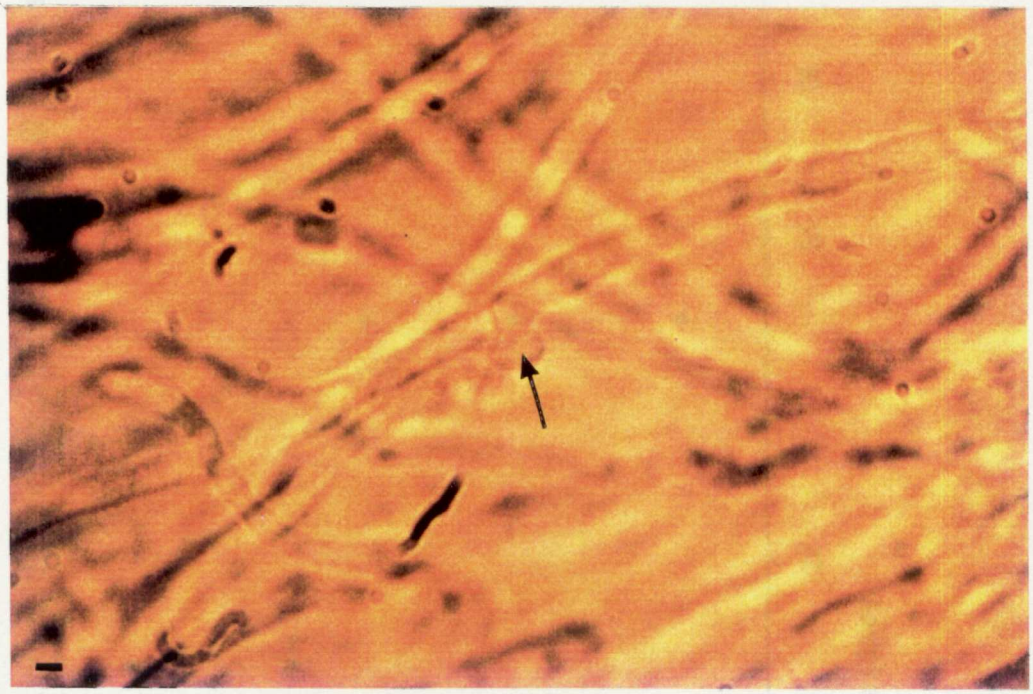


Fig 2.9. Phase contrast micrograph of the secondary phase of *C. perniciosa*. Note the presence of clamp connections (⟶) between septal cells. Scale bar equals 5 µm.

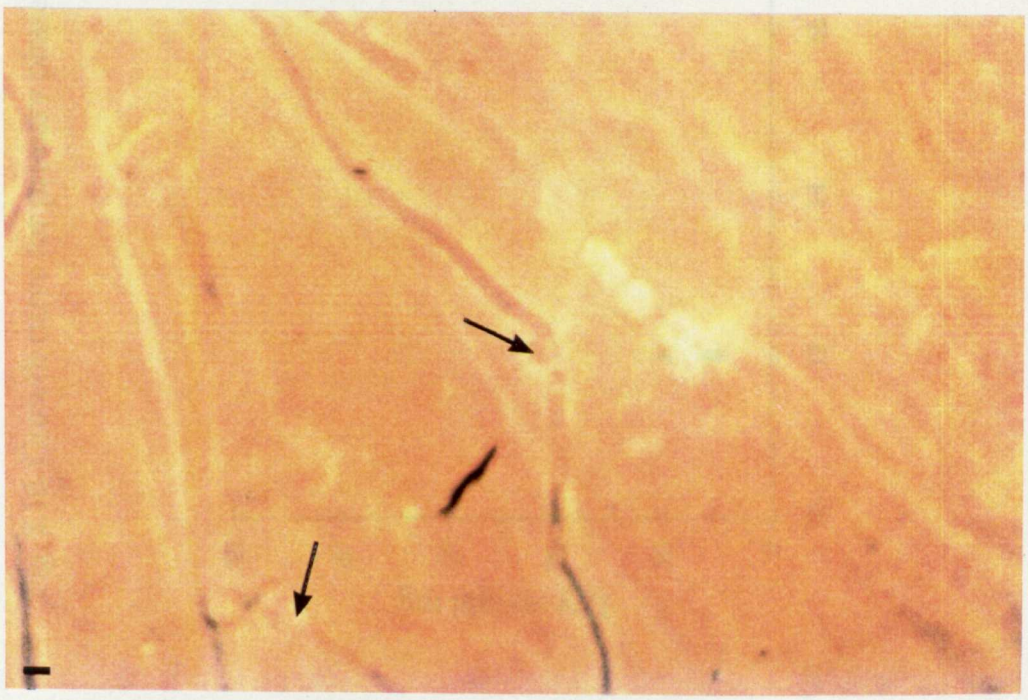


Fig. 2.10. Growth curve for primary phase *C. perniciosa* grown in potato dextrose liquid medium. For full details see 2.1.1.

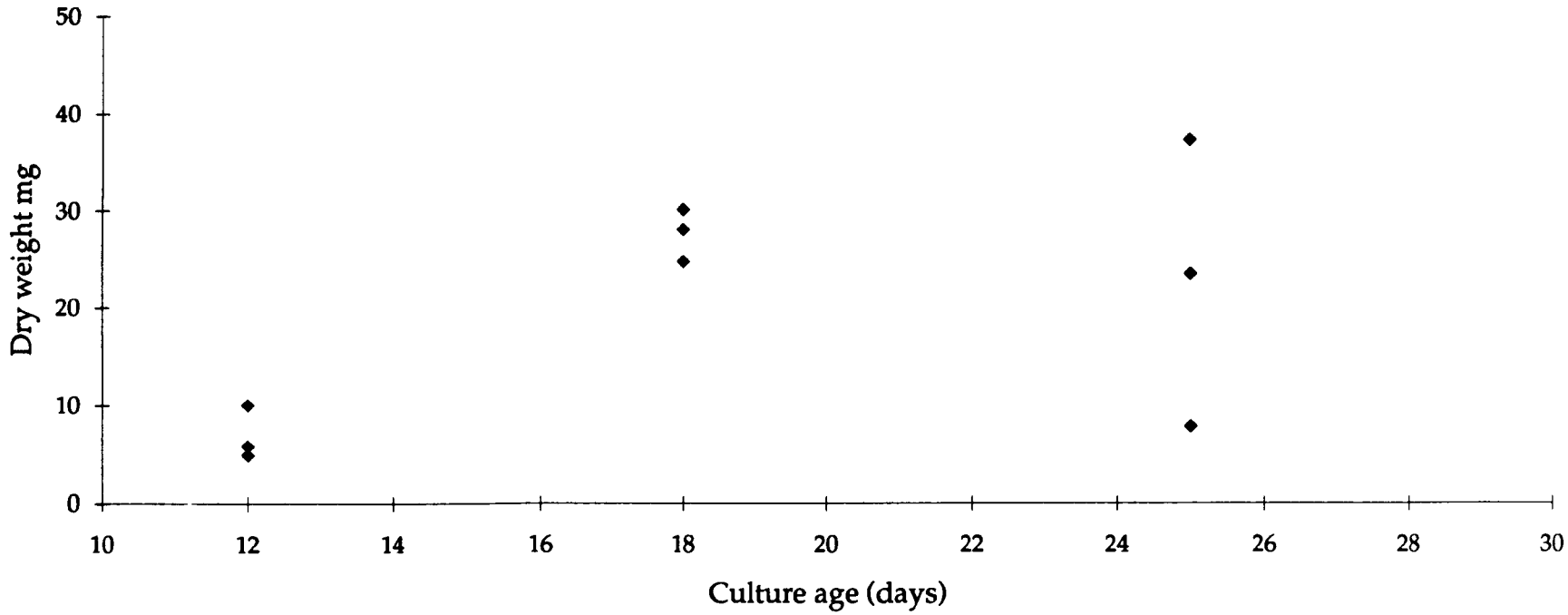


Fig. 2.11. Growth curve for secondary phase *C. perniciosa* grown in potato dextrose liquid medium. For full details see 2.1.1.

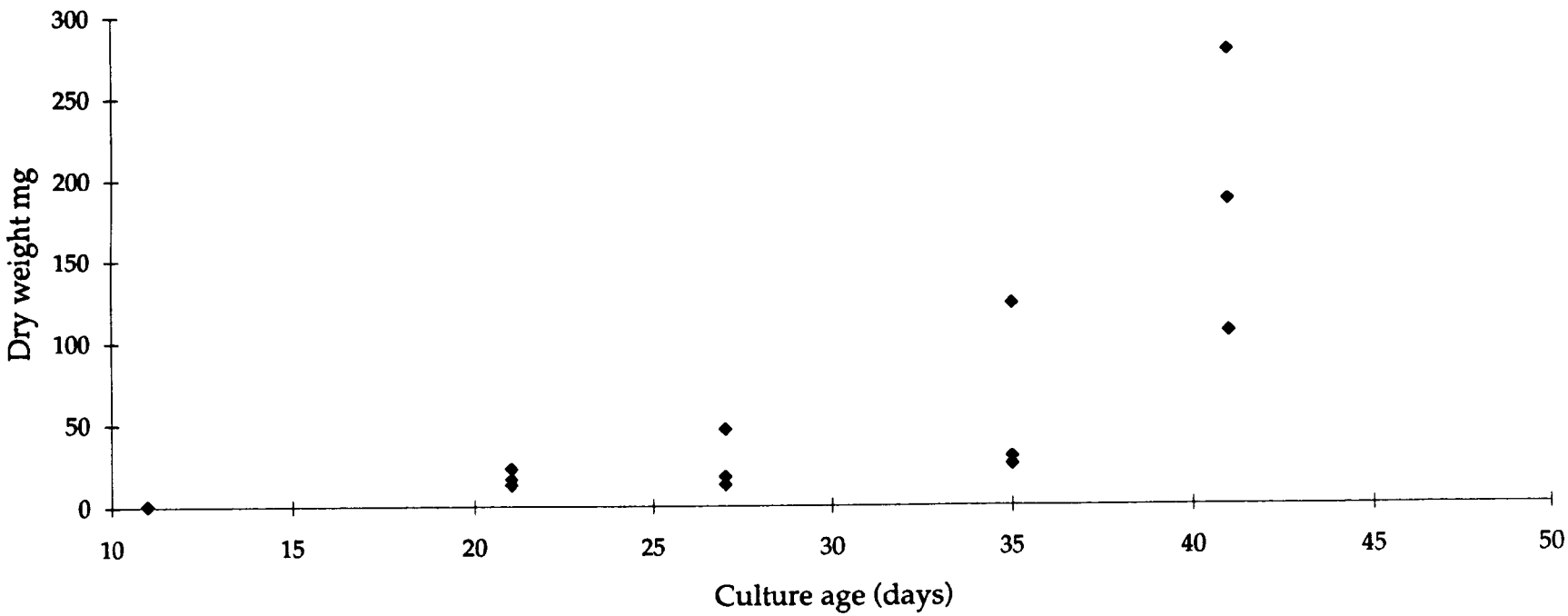


Table 2.1. The length of time primary phase *C. perniciosa* mycelia were maintained in culture as indicated by the presence or absence of clamp connections (see section 2.1.2).

	Sample							
Culture age (days)	1	2	3	4	5	6	7	8
7	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X
35	X	<5	X	<5	X	X	X	X
42	√	√	√	<5	√	√	√	√

X- complete absence of clamp connections. The sample was therefore considered to be composed entirely of primary phase mycelia.

<5- less than 5 clamp connections observed throughout all replicate views of the sample. The primary phase mycelia were therefore considered to have switched to the secondary phase.

√- numerous clamp connections seen. The sample was therefore considered to be composed entirely of secondary phase mycelia.

Chapter 3

The development of chitin and mannan estimation procedures

3.1 Introduction

Combined mannan and chitin analyses have been used as indicators of fundamental developmental changes in many fungi. Whipps *et al.* (1980) compared the growth and development of *Puccinia hordei* Otth. on barley using a range of histochemical and biochemical techniques. The authors found chitin and mannan concentrations agreed with histological observations of infection, and changes in the ratio of mannan to chitin proved to be good indicators for fungal growth and development in barley.

Whipps *et al.* (1980) found that sporulation in *P. hordei* was accompanied by a significant increase in the ratio of mannan to chitin, and that this could be used as an indicator for fungal development. If the ratio between two components is significantly different in mycelia from two life cycle phases, then the relative proportions of the two different mycelia can be calculated in a mixture of the two. In order to calculate this, three items of information must be known. Firstly, the ratio of the two components in both growth phases must be known. Secondly, the total ratio of the components in the unknown mixture of mycelial types must also be known. And thirdly, the level of difference between the component ratios for the two mycelial phases must be statistically significant. The statistical model which would allow the calculation of the concentration of primary and secondary phase *C. perniciosa* in a mixture of the two is shown below.

$$x = \frac{r_0 - r_2}{r_1 - r_2}$$

r_0 = Overall ratio

r_1 = Mannan chitin ratio in the primary phase

r_2 = Mannan chitin ratio in the secondary phase

x = Proportion of primary phase

1-x = Proportion of secondary phase

This model is the same as that used to calculate the allelic composition (1 gene, 2 alleles) of two parent populations from the allelic composition of a daughter population. The formula is therefore commonly used in population biology (John Rogers pers. comm., Dept. Genetics and Microbiology, University of Liverpool, UK). The mathematical problem of apportioning the relative concentrations of primary and secondary phase *C. perniciosa* in a mixture of the two is identical to calculating the allelic composition of two distinct parent populations before the commencement of intermixing.

The aim of this chapter was to investigate whether mannan to chitin ratios could be used to investigate the relative distribution of primary and secondary phase *C. perniciosa* in infected cocoa. The first stage of this procedure was to produce viable and sensitive analytical procedures for measuring mannan and chitin, and then to determine whether potentially useful ratio differences were present. Other workers have used sugar analysis for different but related purposes, their work is outlined below.

Whipps *et al.* (1982) looked at the potential uses of a range of chemical markers to study the growth and development of various fungi in a variety of infected tissues. The authors found chitin to be a useful marker for growth and development in mycorrhizal associations. They also suggested that chitin and mannan contents may be used as indicators of fungal growth in Ericaceous mycorrhizal infections. Concentrations of chitin and mannan were also found to increase in line with growth and development of *Puccinia poarum* Niels. in *Poa pratensis* L. and *Tussilago farfara* L. Later, Whipps *et al.* (1985) found that prior to sporulation of *P. hordei* on barley, there was a significant shift in the ratio of mannan to chitin. The authors concluded that for the calculation of relative but not absolute levels of fungal infection, chitin and mannan assays were of great value for the estimation of fungal biomass.

Katohda *et al.* (1984) found that the mannose content of the cell wall in *Saccharomyces cerevisiae* was about ten times the concentration of the spore cell wall. The level of glucosamine was about five times greater in the spore walls than in the vegetative cell walls and the level of glucose in the cell walls was about half the level of that in the spores. In addition, uronic acid

made up 3 % (w/w) of the spore wall and did not occur in the vegetative walls. The authors did not look at ratios of neutral and amino sugars, but the differences in ratios between mannose and glucose would probably have allowed the quantification of the relative concentrations of sporulating and non-sporulating biomass. Likewise, ratios between glucose, mannose, glucosamine, and uronic acid in various combinations probably would not have allowed the calculation of the relative concentrations of vegetative and sporulating biomass. However, subjective judgements concerning the relative concentrations of vegetative and sporulating biomass could still be made. But unfortunately, in a complex plant environment glucose would not have allowed a useful ratio to be calculated, but other ratios may have proved useful.

Malowitz & Pisano (1982), while investigating changes in cell wall composition during antibiotic production in *Paecilomyces persicinus* P-10 M1, found shifts in neutral and amino sugar concentrations during ageing. They found that the level of chitin increased while that for glucose decreased. Thus, a developmental change, cephalosporin C production, was accompanied by a shift in cell wall composition.

Shibata *et al.* (1986) investigated the cell wall components of morphological mutants of *Rhizoctonia solani* Kühn R-44. They found major differences in glucose mannose ratios in cell wall fractions of the strain and a morphological mutant. Unfortunately, because of the ubiquitous nature of glucose the results were not directly applicable to the work in this thesis. However, the important point was that substantial differences were found in sugar ratios between different morphological variants in a key cellular component.

Many workers have looked at the relative proportions of sugars to aid in strain typing (Lyon & Domer, 1985). Such strain typing often involves isolating a specific component for analysis, such as glycoproteins (Oliva *et al.*, 1985), or cell wall fragments (Lyon & Domer, 1985). Less common was strain typing in which the whole cell wall or organism was analysed.

Many other workers have found differences in mannan and other sugar concentrations in different life cycle phases. For example, Bartnicki-Garcia (1968) found that the yeast form of *Mucor rouxii* possessed five to six times as much mannan than the mycelial form. Re-working this data indicated that the mannan to chitin ratio in the yeast form was 1.06:1 whereas in the hyphal form the ratio was 5.9:1. Such differences in mannan to chitin ratios may have been sufficient to allow the calculation of the relative concentrations of the two fungal forms. Unfortunately, insufficient data was available to calculate the required difference in ratios for the procedure to have been useful. But, a subjective judgement of the relative proportions of the two morphologies in a mixture could still have been made.

Problems associated with chitin analysis

The validity of a chitin assay for estimating fungal biomass has been questioned by some workers, most notably by Sharma *et al.* (1977). Thornton *et al.* (1991) also reiterated the drawbacks associated with the currently used procedures as well as with chitin assays in general. The authors outlined three areas of criticism against the currently used colorimetry based assays. The first two criticisms (a and b, outlined below) were against the biochemical bases of the various assays. The final criticism (c, outlined below) was against the mycological validity of any chitin assay.

a) The assays were confused by galactosamine present in plant tissues (Racusen & Foote, 1974; Thornton *et al.*, 1991). The commonly used assays were incapable of distinguishing between the different hexosamines and therefore the assays detected both galactosamine and glucosamine (the hydrolysed product of chitin). Therefore, in natural extracts containing a variety of hexosamines, a false estimate of the chitin concentration resulted. Thus, any estimate of fungal biomass based upon the concentration of chitin in a sample would also be inaccurate. Kaminskyj & Heath (1982) and Mayama *et al.* (1975) re-emphasised the problems associated with the unspecificity of the commonly used assays. These criticisms could not be answered using the existing colorimetry based chitin estimation procedures. It was not possible to distinguish between glucosamine and many other interfering factors, for example, galactosamine. In addition, pigments of plant origin or particulate matter may have unduly

affected a colorimetry based assay measuring absorbence at 653 nm (Britton pers. comm. Dept. Biochemistry, University of Liverpool, UK).

b) The commonly used alkali extraction procedure did not proceed to completion (Thornton *et al.*, 1991). Thus, the value for chitin obtained from a sample reflected variations in reaction conditions. Whipps & Lewis (1980); Kaminskyj & Heath (1982); Plassard *et al.* (1982) reiterated the problems associated with the commonly used alkali extraction procedure. Without altering the existing extraction protocol the procedure could not be induced to proceed to completion. Other workers (Rocha, 1983) used an acid based extraction procedure which produced more consistent results (Plassard *et al.*, 1982).

c) The mycelial content of chitin was often found to vary during fungal ageing and development (Sharma *et al.*, 1977). Also, the chitin content of a fungus grown in culture did not adequately reflect the concentration of chitin found when the same fungus was grown under different environmental conditions, such as within the host plant. Thus, an estimated value of fungal content in a sample based on a conversion factor obtained from a fungal culture did not accurately reflect fungal content. Whipps *et al.* (1985) countered these criticisms by using a chitin assay for comparative purposes only, rather than as an absolute measure of fungal biomass.

Problems associated with mannan analysis

Mannan concentrations may also fluctuate relative to biomass throughout the fungal life cycle. In addition, the mannan content of a fungus grown in culture may not adequately reflect the content when the fungus is grown under different environmental conditions, such as the host plant. However, a mannan assay may still be used for comparative purposes. Using gas chromatography for mannan analysis eliminates the problems associated with interfering components from the fungus or host, e.g. from hexose sugars.

Chitin estimation procedures

Many researchers have equated the levels of chitin in infected tissues to the level of fungal biomass. Ride & Drysdale (1971) estimated the amount of *Fusarium oxysporum* F. *lycopersici* in infected tomato tissues using a chitin assay. The method of Ride & Drysdale (1971) utilised a colorimetric assay involving 4-dimethylaminobenzaldehyde (Ehrlich's reagent). This method allowed the detection of 5 μg fungus in 1 g fresh weight of tomato. The authors also found that the chitin content in fungal shake cultures of the fungus did not vary significantly with age.

Ride & Drysdale (1972) described an improved chitin estimation procedure based upon the reaction between 3-methyl-2-benzothiazolone hydrozone (MBTH) and the aldehyde derived from glucosamine (2, 5 anhydromannose). The method relied upon the production of amorphous chitosan (deacetylated monomers of chitin) during strong alkali extraction and the monomers were subsequently deaminated and reacted with MBTH to form a chromophore.

The presence of residual acetyl groups in the chitosan rendered portions of it resistant to deamination and thus the true level of chitin was often under estimated. Only a portion of the chitin was deacetylated and therefore the relative level of chitin indicated by the procedure varied significantly with reaction conditions (Plassard *et al.*, 1982). Thus, there was a possibility that the whole methodology may have been overly sensitive to reaction conditions. As only a portion of the chitin monomers (glucosamine) were detected, then the method was less sensitive than one which utilised the same colorimetry step but which assayed for all the glucosamine present. In addition, Plassard *et al.* (1982) found that a method which used a strong acid extraction procedure, for example 6N HCl, yielded approximately 90 % of the chitin present whereas the alkali extraction procedure used by Ride & Drysdale (1972) yielded only 50 %. Rocha (1983) modified the Swift (1973) procedure and produced such a method, see below.

Another problem with the Ride & Drysdale (1972) method was the unspecific nature of the colorimetric reaction. The assay was not specific for glucosamine but detected all hexosamines. Thornton *et al.* (1991) considered this unspecificity to be a problem as plant derived galactosamine was also detected. Since Ride & Drysdale (1972) published their work there have been numerous attempts to improve the effectiveness of the colorimetry based chitin assay. Kaminskyj & Heath (1982) attempted to improve the assay by increasing the effectiveness of the chitin extraction and purification procedures. Many workers (for example, Thornton *et al.*, 1991) claimed the improved assay was more effective. The assay was more effective, but, the same three reservations concerning a chitin assay, as described earlier, still applied.

Lung-chi Wu & Stahman (1975) used a chitin estimation procedure based upon the use of an amino acid analyser. The authors claimed the method was particularly sensitive and reliable. Unfortunately, we did not have access to an amino acid analyser in the department, so the method could not be further investigated.

An alternative technique of chitin estimation was employed by Van Pelt-Heerschap & Sietsma (1990). The authors hydrolysed chitin from the cell walls of *Didymella bryoniae* using a HCl extraction procedure. The glucosamine was then quantified using thin layer chromatography with ninhydrin as the developing agent. As ninhydrin was used in the detection procedure the assay therefore suffered from being relatively unspecific. Ninhydrin labels amino groups and therefore virtually any compound with an amino group will have been labelled. In addition, as all hexosamines are virtually chemically identical then it was unlikely that glucosamine was separated from plant derived galactosamine.

An alternative colorimetric procedure was employed by Chen & Johnson (1983). This methodology involved hydrolysing the chitin content of a fungal/plant sample by refluxing with 6M HCl. The chitin was thereby hydrolysed to glucosamine hydrochloride. The glucosamine hydrochloride then formed a measurable coloured reaction product (a chromogen) in the presence of N-N dimethyl-p-aminobenzaldehyde.

The Boas (1953) method of chitin estimation utilised ion exchange chromatography to remove unwanted potential chromogens and utilised the same colour development step as Chen & Johnson (1983). The methodology promised a great increase in speed over the following Rocha (1983) method, described below, because the eluents produced from an ion exchange clean-up step were neutralised rather than eliminated by evaporation. The acid evaporation step used by Rocha took two days for a batch of 20 samples. The Boas (1953) methodology was unfortunately inherently less sensitive than the following Rocha (1983) procedure because of the post ion exchange neutralisation step described earlier. This step diluted the sample, whereas the sample was concentrated concurrently with acid removal by evaporation in the Rocha (1983) procedure. This ensured the Boas (1953) method was relatively quick, but only about one seventh as sensitive as the Rocha (1983) procedure. For this reason the method was deemed inappropriate for this project.

From the large number of colorimetric chitin assays available, the method used by Rocha (1983) appeared the most appropriate. Rocha (1983) adapted a method for use in cocoa tissue originally devised by Swift (1973). Rocha (1983) utilised the more modern and sensitive Tsuji *et al.* (1969) colorimetry step in replacement of the methodology devised by Elson & Morgan (1933) and utilised by Swift (1973) and others. In addition, an ion exchange clean-up procedure was incorporated to remove many of the potentially interfering components.

Because of the volume of work performed during the testing and eventual production of the new chitin and mannan assays, this chapter was divided into three sections. The first dealt with the testing of the Rocha (1983) chitin estimation procedure. Section 2 dealt with the attempt to develop a chitin and mannan estimation procedure using high performance liquid chromatography. The third section concerned the development of chitin and mannan assays using gas chromatography and mass spectrometry.

3.1.1 The Rocha (1983) method of chitin estimation

3.1.1a Introduction

The aim of this work was to investigate the Rocha (1983) method of chitin estimation to determine whether it was viable as part of a chitin mannan estimation procedure. The basis of the assay was the hydrolysis of chitin to glucosamine and then to glucosamine hydrochloride which was then deaminated, the resultant 2, 5 anhydromannose then reacted with 3-methyl-2-benzothiazolone hydrozone (MBTH) in the presence of $FeCl_3$ and produced a chromogen with an absorbtion maximum at 653 nm. Prior to deamination the samples were cleaned with cation exchange resin.

3.1.1b Methods

Samples of 6 month old cocoa (Amelonado) apical region tissues (meristem, up to and including, 4 cm below the apical bud tip) were freeze-dried, and ground in a mortar and pestle. Portions of the samples (25 mg) were placed in 10 ml volumetric flasks. To each of the 5 replicates, 3 ml of the following concentrations of glucosamine in 6M HCl was added; 100, 80, 60, 40, 20, 10, 5, 0 µg/ml. The volumetric flasks were then sealed and heated at 100°C in a boiling water bath for 6 hours.

The hydrolysates were then filtered with Whatman No° 1 filter paper. The filtrates and washings were recombined and reduced to dryness under reduced pressure at 60°C to remove the HCl. The hydrolysates were resuspended in 3 ml of distilled water and reduced to dryness. The re-suspension and evaporation steps were repeated a further 3 times to ensure complete removal of the HCl. The hydrolysates were resuspended in 3 ml distilled water and pipetted onto 1 cm diameter columns containing 5 ml of Dowex 50 (200-400 mesh) cation exchange resin. The columns were washed with 16.5 ml of distilled water and the glucosamine eluted with 21.5 ml of 2M HCl. The columns were regenerated with 10 ml 2M NaOH followed by 21.5 ml 2M HCl.

The HCl was then removed from the eluents by evaporation as described earlier, before finally being resuspended in 3 ml distilled water. One drop of 0.5% phenolphthalein in

ethanol solution (v/v) was added to the hydrolysates before neutralisation with 1M NaOH and 1% $KHSO_4$ (w/v). The solutions were then made up to 4 ml. Aliquots (1.5 ml) of each hydrolysate were removed and 1.5 ml 5% $NaNO_2$ (w/v) and 1.5 ml of 5% $KHSO_4$ (w/v) added and the mixtures shaken continuously for 15 min.

The hydrolysates were then centrifuged at 1500 G for 5 min and 1.5 ml of each hydrolysate removed and placed into a clean test tube. To each hydrolysate, 0.5 ml of 12.5% (w/v) $NH_4SO_3NH_2$ (ammonium sulphamate) was added. The mixtures were shaken for 5 min. To the hydrolysates, 0.5 ml of 0.5% MBTH (w/v) solution was added and the mixtures heated at 100 °C for 3 min. The hydrolysates were then cooled to room temperature. Aliquots (0.5 ml) of 0.5% $FeCl_3$ (w/v) were then added and the mixtures left to stand at room temperature for 30 min. The absorbence was then read at 650 nm on a LKB Biochrom Ultrospec 4050 spectrophotometer.

Suppliers

Dowex 50, phenolphthalein, MBTH, $FeCl_3$, and glucosamine (as glucosamine hydrochloride) were supplied by Sigma Chemical Company Ltd. HCl, NaOH, $KHSO_4$, and $NaNO_2$ supplied by BDH Chemicals Ltd. Ammonium sulphamate supplied by Aldrich Chemical Company Ltd.

3.1.1c Results and Discussion

The standard curve illustrated in Fig. 3.1 was produced from cocoa tissue spiked with D+ glucosamine in a range of concentrations from 15 to 300 µg/25 mg of dried cocoa.

The standard curve illustrated that a reliable quantification limit for glucosamine in cocoa tissue was about 90-120 µg/25 mg of dry sample. The apparent high background concentration of glucosamine was almost certainly not glucosamine but due to a range of non-hexosamine products liberated during acid hydrolysis. When cocoa was refluxed with a strong acid, a dense purple/black colouration was produced. It was hoped the ion exchange clean-up procedure would have eliminated this problem. Rocha (1983) working with cocoa

did not find this a problem. He estimated the background level of hexosamines to be about 1 μg/25 mg of material. The difference in absolute values between the work shown here and Rocha's (1983) may have been due to differences in experimental material. Rocha used cocoa cultivar Na32 X UIT1, and IMC67. The material for this work originated from the cultivar Amelonado.

All assays based upon the existing colorimetric procedures are flawed because of their relative unspecificity. Cocoa presented particular problems for colorimetric based assays because the tissues contained a wide variety of coloured components which interfered with the assays. It was possible that a more comprehensive series of clean-up steps may have improved the assay, but the basic flaw common to all colorimetric assays for glucosamine would still have remained. Any accurate measure of chitin content must be specific for glucosamine and must therefore be capable of discriminating between glucosamine, galactosamine, and other potentially interfering components. The remainder of this chapter was therefore concerned with the search for, and eventual production of, a method which eliminated the biochemical based problems of a colorimetric chitin assay. The problems centred on unspecificity, insensitivity, and lack of reproducibility. Such an ideal chitin estimation procedure would also be capable of measuring mannan concurrently, if not simultaneously.

3.1.1d Conclusions

The Rocha (1983) modification of the Swift (1973) procedure was not a viable method for this project. The central problem with the procedure was the production of a dense colouration from cocoa tissue. This colouration completely obscured the chromophores produced from glucosamine. Therefore, the methodology was inadequate on the grounds of insensitivity and probable unspecificity for glucosamine.

3.2.1 A chitin and mannan estimation procedure using HPLC

3.2.1a Introduction

High performance liquid chromatography (HPLC) is a procedure which separates compounds in the liquid phase on the basis of polarity, molecular weight, and hydrophobic/hydrophilic interactions. A range of specialist detectors are also available for HPLC.

Sugars absorb radiation at round 190 nm. Unfortunately, all solvents suitable for sugars and available for HPLC absorbed strongly in the 180-220 nm region of the spectrum. Thus, sugars required derivatisation to enable them to absorb at longer wavelengths.

Most sugar analysis procedures listed in the literature used a refractive index (RI) detector. A RI detector was not suitable for use in cocoa because they are prone to background interferences. UV detectors are very much more sensitive than RI detectors. The use of an ultraviolet (UV) detector monitoring at a specific wavelength would have suffered far less from interference since only a relatively small number of compounds absorb at any given wavelength. This contrasts with an HPLC system equipped with a refractive index detector which identifies compounds on the basis of chromatographic separation only.

Many workers have used fluorescence detectors (Hjerpe *et al.*, 1980; Takemoto *et al.*, 1985; Hase *et al.*, 1981; Mopper & Johnson, 1983). Unfortunately, a fluorescence detector was unavailable for use in the department. It was therefore decided to develop a method which utilised UV detection. Below is a brief resume of the most promising derivatisation procedures that may have formed the basis of an improved method of analysis.

Batley *et al.* (1982) developed an analysis system for aldose sugars using reversed phase HPLC and UV detection at 254 nm. The method appeared quick and relatively straightforward. However, in complex plant materials the results from a detector operating at a wavelength of 254 nm may have suffered from the effects of background interferences (Britton pers. comm.)

Bjorkqvist (1981) described a method for producing phenylisocyanate derivatives of various carbohydrates and their separation using a reversed phase system with a detection wavelength of 240 nm. The method was also adapted for the detection of polyols in mammalian tissues (Dethy *et al.*, 1984). Hjerpe *et al.* (1980) used a procedure for separating galactosamine and glucosamine, the method appeared over complicated and insufficiently sensitive for our needs, the detection limit was more than 1 μg/mg of sample. In addition, the results from a detector measuring absorbence at 240 nm may have suffered from the effects of background interferences.

Lehrfeld (1976) produced a method for separating carbohydrates as per-benzoate esters using HPLC with a silica column and UV detection at 275 nm. The derivatisation procedure was relatively quick and efficient, and the separation between carbohydrates on the resultant chromatograms appeared good. The method appeared adaptable for use with amino sugars. Oshima & Kumanotami (1983) adapted Lehrfeld's (1976) methodology for use in a wider range of carbohydrates including amino sugars. The method appeared to be a suitable basis from which to develop a new and highly sensitive analytical procedure for the detection and quantification of chitin and mannan in cocoa infected with *Crinipellis perniciosa* and other diseased plant tissues.

The basis of Oshima & Kumanotami's (1983) methodology

The chitin and mannan were hydrolysed from the cell walls of the fungus using 2M trifluoroacetic acid. The corresponding sugar monomers (glucosamine and mannose respectively) were then reduced to the corresponding alditols (sugar alcohols) using sodium borohydride. In the process of reduction the borate produced from the sodium borohydride formed complexes with the alditols and thus prevented further derivatisation. Borate was removed from the complexes using a mixture of hydrochloric acid and methanol. The borate was then removed from the mixture by evaporation as the trimethyl ester of borate. The alditols were then derivatised to their corresponding benzoate esters using benzoic anhydride, with 4-dimethylaminopyridine and pyridine acting as catalysts. The samples were then cleaned by partitioning between chloroform and water.

3.2.1b Methods

Samples of 6 month old Amelonado cocoa apical region tissue (meristem, plus up to and including, 4 cm below the apical bud tip) were freeze-dried and then ground in a mortar and pestle. Portions (10 mg) of the samples were transferred to methylating tubes with PTFE cap liners. Aliquots (2 ml) of 2M trifluoroacetic acid were added and the mixtures autoclaved for 1 hour at 120°C. After extraction the trifluoroacetic acid was evaporated off using a stream of nitrogen gas with gentle heating from the base. After evaporation, 2 ml of distilled water was added to the samples. Aliquots (2 ml) of distilled water were also added to a series of standards consisting separately of 10 mg of; glucosamine, galactosamine, glucose, mannose, and fructose. The samples were then vortex mixed for 1 min before 50 mg of $NaBH_4$ was added and the samples allowed to stand at 40°C for 2 hours, with occasional shaking.

The reduced extracts and standards were acidified to pH 2.5-3 using 2M HCl, then dried at 20°C under vacuum. Aliquots (3 ml) of redistilled methanol were added to the samples and subsequently evaporated to dryness under vacuum. The samples were dried further at 50 °C in a vacuum oven for 2 hours. The samples were then derivatised by adding 200 mg of benzoic anhydride, 100 mg of dimethyl amino pyridine, and 2 ml of dried pyridine. The samples were heated in a water bath for 2 hours at 50 °C with continuous stirring. One ml of redistilled methanol was added to each sample, which were then dried in a vacuum oven at 60 °C. After drying each sample was resuspended in 2 ml of chloroform and partitioned upon the addition of 2 ml of distilled water. The upper aqueous phase was removed and discarded. The lower chloroform phase was filtered using Whatman No° 1 filter paper suspended over a scintered glass funnel. The filtrates were transferred to 6 ml capacity screw top vials and evaporated to dryness under a stream of nitrogen gas. The samples were resuspended in 1 ml of chloroform prior to analysis.

HPLC procedure

HPLC was performed with a Waters 600E Control Unit, a Waters 600 Multisolvent Delivery System, and a Waters 990 Photodiode Array Detector. Data was collected, stored, and analysed on a NEC Powermate 2 Advanced Personal Computer. Separation of components

was achieved using a Phase Separations S5W plain silica column (5 μm silica, 25 cm length with an internal diameter of 4.6 mm). An isocratic mobile phase was used and consisted of hexane, dioxane, and dichloromethane (73:18:9).

Suppliers

Trifluoroacetic acid, $NaBH_4$, all sugars and amino sugars were supplied by Sigma Chemical Co. Ltd. Benzoic anhydride, dimethyl amino pyridine, and pyridine supplied by Lancaster Synthesis Ltd. Methylating tubes (sold as culture tubes), HCl and chloroform supplied by BDH Chemicals Ltd. Hexane, dioxane, and dichloromethane supplied by Fisons Scientific Ltd. The 6 ml capacity screw top bottles were supplied by S. Murray & Co.

3.2.1c Results and Discussion

The data illustrated in Fig. 3.2 demonstrated that mannose had an elution time of approximately 15 min whilst glucosamine had an elution time of approximately 17.9 min and therefore both mannose and glucosamine were adequately resolved and quantified. The separation for glucose (Fig. 3.3) demonstrated that it had an elution time of approximately 14.4 min, which was very similar to the elution time of mannose. Therefore, when glucose was present in a large excess with respect to mannose the two peaks could not be resolved. Thus, mannose could not be quantified in the presence of relatively high levels of glucose. In addition, when glucose was present in a large excess with respect to glucosamine, then neither could be resolved or quantified.

The chromatogram shown in Fig. 3.4 was obtained from cocoa apical region tissue treated in accordance with the analysis procedure. The chromatogram clearly illustrated the effects of one component when in large excess to the others. The effect was to introduce a large amount of peak tailing which rendered any form of peak identification and therefore quantification impossible. The component which caused this effect in cocoa tissue hydrolysate was almost certainly glucose. The absorbtion spectrum of the component (illustrated at the top of Fig. 3.4) indicated the component was a hexose sugar. This component eluted in the same region as glucose and therefore it was reasonable to assume that the component was glucose. Nuclear

magnetic resonance analysis would need to be performed to confirm the identification. The glucose was probably produced from hydrolysed cell walls and starch.

3.2.1d Conclusions

The HPLC method as described was not suitable to form the basis of a more efficient chitin/mannan estimation procedure. The main problem with the procedure was the swamping effect by various hexose sugar components on glucosamine and mannose. A series of modifications in chromatographic and experimental conditions were attempted in order to remove or ameliorate the problem, all without success.

3.3.1 Preliminary procedures for chitin and mannan estimation using gas chromatography

3.3.1a Introduction

Previous work (3.1.1) indicated there were insuperable problems associated with the existing chitin assay when used with cocoa tissue. In addition, the attempts to develop a combined chitin and mannan assay using HPLC produced poor results (3.2.1). Therefore, it was decided to pursue a new line of investigation using gas chromatography. Gas chromatography has several advantages over other sugar analysis techniques. The technique is currently one of the most sensitive analytical procedures available, especially if a mass spectrometer is used as the detector. The technique also produces highly specific results and would be capable of discriminating between glucosamine, galactosamine, and other potentially interfering components present in cocoa tissue.

Gas chromatography separates volatile compounds on the basis of polarity, molecular weight and structure. As many of nature's compounds are not volatile within the normal operating temperature range of a GC, it would be necessary to increase the volatility of the compounds under investigation. Increasing the volatility of a compound is normally achieved by attaching volatile side groups, such as acetyl or trimethylsilyl groups, to the parent molecule.

Whipps *et al.* (1980; 1982; 1985) in their investigations using mannan and chitin ratio analysis used gas chromatography for the estimation of mannan, but not for chitin. Since Whipps *et al.* (1980; 1982; 1985) conducted their work there have been numerous advances in gas chromatograph design, column technology, and derivatisation procedures. Other workers have used techniques similar to the Whipps *et al.* (1980) adaption of the Jones & Albersheim (1972) procedure. The fundamental point of difference in all the GC based sugar analysis procedures was the nature of the volatile derivative used for analysis. Most used acetyl derivatives (Whipps *et al.*, 1980 onwards), a few used trimethylsilyl derivatives (Bradbury, 1981), or less commonly, trifluoroacetyl (Haga & Nakajima, 1988), or methyl derivatives.

It was decided to use acetyl derivatives as other workers have used the same derivatives in similarly complex plant/fungal tissues (Jones & Albersheim, 1972; Whipps *et al.*, 1980). Since Whipps *et al.* (1980 onwards) conducted their work there have been advances in acetylation procedures. The work of Whipps *et al.* (1980 onwards) required acetylation conditions with temperatures in excess of 100 °C, for more than 12 hours. Whiton *et al.* (1985) produced a method based on the use of N-methylimidazole as an acetylation catalyst. The catalyst promised to cut the derivatisation time to 45 min at a temperature of 37 °C. It was decided to investigate this procedure. Unfortunately, there were a series of errors associated with the published procedure, the most notable being with the acetylation (derivatisation) method. The following work concerned the first five attempts to produce chitin and mannan estimation procedures; they are discussed collectively after the presentation of the final method.

It was not possible to produce a combined mannan and chitin extraction procedure because of the differences in polymer resistance to strong acids. Chitin has proved to be a very much more resistant polymer than mannan, it required upwards of 6 hours in 6 M HCl at 100 °C for complete hydrolysis (Swift, 1973; Plassard *et al.*, 1982), whereas mannan was hydrolysed in 2 hours at 120 °C with 2M trifluoroacetic acid (Whipps *et al.*, 1982). Therefore, the following initial work concentrated on producing viable derivatisation, clean-up, and analysis procedures. The extraction procedures could then be tailored to suit the different requirements of chitin and mannan afterwards. Therefore, the following preliminary work was performed on the monomers, glucosamine, and mannose, and also on glucose.

3.3.1b Methods of chitin and mannan estimation

Method 1

The following procedure was the method recommended by Whiton *et al.* (1985).

Approximately 125 µg of mannose, glucose and glucosamine were added to separate 6 ml capacity screw top vials. To the sugars, 250 µl of distilled water and 60 µl of $NaBH_4$ solution containing 10 mg/ml of $NaBH_4$ in methylimidazole were added. The vials were heated at 37

°C for 90 min. To the tubes, 20 µl of glacial acetic acid and 600 µl of acetic anhydride were added. The mixtures were then heated at 37 °C for 45 min. The samples were cooled in an ice bath and 800 µl of cold 80 % (v/v) ammonium hydroxide and 1 ml of chloroform were added and the resulting mixtures vortex mixed and poured onto hydrophilic columns. The derivatised sugars were eluted with 2 ml of chloroform. Six hundred microlitres of 1M HCl was added to the eluents and the mixtures once again vortex mixed and poured onto hydrophilic columns. The derivatised sugars were eluted with 2 ml of chloroform and the eluents reduced to dryness. Prior to analysis they were redissolved in 500 µl of ethyl acetate.

Method 2

The following procedure utilised a drying step after reduction but before acetylation. This adaption was recommended by Whiton *et al.* (1985) in order to produce cleaner chromatograms.

Approximately 125 µg of mannose, glucose and glucosamine were added to separate 6 ml capacity screw top vials. To the sugars, 250 µl of distilled water and 60 µl of $NaBH_4$ solution containing 10 mg/ml of $NaBH_4$ in methylimidazole were added. The vials were heated at 37 °C for 90 min. To the tubes, 20 µl of glacial acetic acid was added. The solutions were then reduced to dryness in a vacuum oven at room temperature. The samples were then resuspended in 60 µl of methylimidazole. Six hundred microlitres of acetic anhydride was added to the samples. The mixtures were then heated at 37 °C for 45 min. The samples were cooled in an ice bath and 800 µl of cold 80 % (v/v) ammonium hydroxide and 1 ml of chloroform were added and the resulting mixtures vortex mixed and poured onto hydrophilic columns. The derivatised sugars were eluted with 2 ml of chloroform. Six hundred microlitres of 1M HCl was added to the eluents and the mixtures once again vortex mixed and poured onto hydrophilic columns. The derivatised sugars were eluted with 2 ml of chloroform and the eluents reduced to dryness. Prior to analysis the samples were redissolved in 500 µl of ethyl acetate.

Method 3

The following procedure eliminated the hydrophilic columns from the method. The hydrophilic columns were only needed to produce the cleanest possible chromatograms, therefore they presented an unnecessary complication when the procedure was only being tested for the ability to derivatise sugars.

Approximately 125 µg of mannose, glucose and glucosamine were added to separate 6 ml capacity screw top vials. To the sugars, 250 µl of distilled water and 60 µl of $NaBH_4$ solution containing 10 mg/ml of $NaBH_4$ in methylimidazole were added. The vials were heated at 37 °C for 90 min. To the tubes, 20 µl of glacial acetic acid and 600 µl of acetic anhydride were added. The mixtures were then heated at 37 °C for 45 min. The samples were cooled in an ice bath and 800 µl of cold 80 % (v/v) ammonium hydroxide and 1 ml of chloroform were added and the resulting mixtures vortex mixed. The resulting mixtures were allowed to settle and the aqueous phases removed and discarded. Six hundred microlitres of 1M HCl was added to the chloroform phases and the mixtures once again vortex mixed and allowed to partition. The aqueous phases were removed and discarded. The chloroform phases were reduced to dryness. Prior to analysis the samples were redissolved in 500 µl of ethyl acetate.

Method 4

This procedure utilised a drying step after reduction but before acetylation and also eliminated the hydrophilic columns. The drying modification was recommended by Whiton *et al.* (1985) and the hydrophilic columns were considered unnecessary.

Approximately 125 µg of mannose, glucose and glucosamine were added to separate 6 ml screw top vials. To the sugars, 250 µl of distilled water and 60 µl of $NaBH_4$ solution containing 10 mg/ml of $NaBH_4$ in methylimidazole were added. The vials were heated at 37 °C for 90 min. To the tubes, 20 µl of glacial acetic acid was added. The solutions were then reduced to dryness in a vacuum oven at room temperature. The samples were then resuspended in 60 µl of methylimidazole. Six hundred microlitres of acetic anhydride was added to the samples. The mixtures were then heated at 37 °C for 45 min. The samples were

cooled in an ice bath and 800 µl of cold 80 % (v/v) ammonium hydroxide and 1 ml of chloroform were added. The resulting mixtures were vortex mixed. The mixtures were allowed to partition and the aqueous phases removed and discarded. Six hundred microlitres of 1M HCl was added to the mixtures and they were once again vortex mixed and allowed to partition. The aqueous phases were removed and discarded. The chloroform phases were reduced to dryness. Prior to analysis the samples were redissolved in 500 µl of ethyl acetate.

Method 5

The method used significantly higher reagent quantities to ensure that all were present in sufficient concentrations. The hydrophilic columns were also eliminated from the procedure.

Approximately 1 mg of mannose, glucose and glucosamine were added to separate 6 ml capacity screw top vials. To the sugars, 250 µl of distilled water and 600 µl of $NaBH_4$ solution containing 10 mg/ml of $NaBH_4$ in methylimidazole were added. The vials were heated at 37 °C for 90 min. To the tubes, 20 µl of glacial acetic acid and 1 ml of acetic anhydride were added. The mixtures were then heated at 37 °C for 45 min. The samples were cooled in an ice bath and 800 µl of cold 80 % (v/v) ammonium hydroxide and 1 ml of chloroform were added and the resulting mixtures vortex mixed. The resulting mixtures were allowed to settle and the aqueous phases removed and discarded. Six hundred microlitres of 1M HCl was added to the chloroform phases and the mixtures once again vortex mixed and allowed to partition. The aqueous phases were removed and discarded. The chloroform phases were reduced to dryness. Prior to analysis the samples were redissolved in 500 µl of ethyl acetate.

3.3.1c Discussion and Conclusions

After five variations of the experimental protocol, help was sought from R.S. Whiton. Unfortunately, he could not be traced. Contact was then made contact with Dr. Fox (Dept. Microbiology and Immunology, University of South Carolina, USA) who was also a member of the team which produced the paper in question. Dr. Fox admitted that there were many problems associated with the derivatisation procedure and he recommended a different

acetylation procedure that he had been using routinely for many years. There were further problems associated with this newly recommended procedure, which were identified and solved. In retrospect, there were three major problems associated with the methods published by Whiton *et al.* (1985), these were:-

a) Adding 20 µl of acetic acid may have decomposed $NaBH_4$, but the borate would not have been removed. It was essential that borate was removed from the reaction mixture because it formed complexes with the alditols and hexosaminitols and thus prevented derivatisation. In the following new chitin and mannan assays, acetic acid and methanol were added to the reduced mixture. The acid broke down the complexes and the methanol then reacted with the borate and formed a volatile trimethyl ester which was subsequently removed by evaporation.

b) Adding acetic anhydride to an aqueous solution produced acetic acid. Therefore, no acetylation occurred because the acetylating agent was destroyed on contact with the water, the catalyst (methylimidazole) was presumed to prevent this reaction from proceeding. Therefore, for the new chitin and mannan assays, the mixtures were assiduously dried prior to acetylation.

c) The acetylation temperature was insufficient for the reaction to proceed. Temperatures above 100 °C and reaction times in excess of 12 hours are normally required for acetylation (Britton pers. comm.). Thus, the recommended acetylation conditions of 37 °C for 45 min were inadequate to acetylate the sugars, for the final chitin and mannan assays, the mixtures were acetylated for 16 hours at 100 °C.

3.3.2 The new chitin and mannan estimation procedures

3.3.2a Introduction

The following work concerned the finalised methods for chitin and mannan estimation. The chitin estimation procedure centred on the production of acetylated sugar derivatives. The method also used a hydrochloric acid extraction step and flame ionisation and mass spectrometer detectors. The mannan estimation method was based on the chitin estimation procedure and used the mannan extraction step developed by Whipps *et al.* (1980).

3.3.2b Methods

Chitin assay

Freeze-dried plant and fungal samples (detailed below) were ground in a mortar and pestle. The Rocha (1983) chitin extraction procedure was used. Portions (1 mg) of each sample were transferred to glass marble stoppered Pyrex test tubes. After the addition of 3 ml of 6M HCl, the samples were heated at 100°C in a boiling water bath for 6 hours. The HCl was blown off overnight under a stream of nitrogen gas. During the blow-down procedure the test tubes were gently heated from below at 40 °C using a block heater.

To the dry extracts, 10 µg of the internal standard methylglucamine dissolved in 2 ml of distilled water was added. Fifty microlitres of sodium borohydride solution (100 mg/ml) was added to reduce the hexosamines to hexosaminitols. The mixtures were heated at 37 °C in a water bath for 90 min. Aliquots (2 ml) of acetic acid redistilled methanol (1:200 v/v) was added to the mixtures which were then centrifuged at 2500 rpm in a MSE Centaur 2 bench centrifuge for 20 min to remove particulate matter. The supernatants were pipetted into 6 ml capacity screw top vials and the pellets resuspended in 1 ml of distilled water. The mixtures were centrifuged again, and the respective supernatants recombined.

The samples were transferred to a vacuum oven heated to 50 °C with a vacuum of 750 mm of mercury. The samples were evaporated to dryness and a further 2 ml of the acetic acid methanol mixture was added (1:200 v/v). Once again, the samples were evaporated to dryness. The addition and drying steps were repeated a further 3 times to make a total

addition of acetic acid methanol of 10 ml. The acetic acid mixture was added to destroy any excess sodium borohydride and to remove borate as the corresponding trimethyl ester. After the final addition of acetic acid methanol the oven temperature was raised to 60 °C. The extracts were allowed to remain in the oven for a further 3 hours to remove any last traces of moisture.

After cooling, 300 µl of acetic anhydride was added to acetylate the hexosamitols. The vials were flushed with nitrogen gas prior to sealing. The vials were heated in a Grant BT4 block heater at 100°C for 15 hours. The acetylated samples were cooled in an ice bath and 0.75 ml of distilled water was added to destroy any excess acetic anhydride. The samples were allowed to stand in the ice bath for 30 min. Redistilled chloroform (1 ml) was added to each of the mixtures which were then shaken and the aqueous phases removed and discarded. To the remaining chloroform phases, 0.8 ml of 80 % (v/v) ammonium hydroxide was added. The mixtures were shaken and the aqueous phases removed and discarded. The chloroform phases were dried by passing through anhydrous magnesium sulphate columns. The acetylated amino sugars were eluted by washing the columns with 2 ml of redistilled chloroform with the eluents being collected in 6 ml capacity screw top vials. The samples were then dried in a vacuum oven at room temperature.

Estimation of the percentage recovery for the procedure

Samples containing 5 µCi of C^{14} labelled D+ glucosamine and 1 mg of D+ glucosamine, acting as a carrier, were reduced and derivatised in accordance with the chitin estimation procedure described in the previous paragraphs. As radiolabelled chitin was unavailable the percentage recoveries could only be estimated for the procedure excluding the hydrolysis step. Counts per min corrected for background levels were measured in the starting solutions, after acetylation, and in the final derivatised solutions.

Estimation of the relative response factor for the flame ionisation detector

In order to ensure the detector response was linear across probable internal standard to sugar concentrations, a series of standards with differing ratios of methylglucamine to glucosamine

were derivatised using the chitin analysis procedure. The ratios analysed were 7, 1 and 0.2 methylglucamine : glucosamine

Mannan assay

Freeze-dried plant and fungal samples were ground in a mortar and pestle and extracted using the Whipps *et al.* (1980) mannan extraction procedure. Portions (1 mg) of the plant and fungal samples were transferred to methylating tubes with PTFE cap liners. Two millilitres of 2 M trifluoroacetic acid was added and the mixtures autoclaved for 1 hour (115 °C). The samples were then treated in accordance with the chitin analysis procedure. Sedoheptulose was used as the internal standard in place of methylglucamine.

Estimation of the relative response factor of the flame ionisation detector for the mannan assay

In order to ensure the detector response was linear across probable internal standard to sugar concentrations, a series of standards with differing ratios of sedoheptulose to mannose were derivatised using the mannan analysis procedure. The ratios analysed were 5, 1 and 0.2 sedoheptulose : mannose

Gas chromatography and mass spectrometry

Separation and quantification of chitin and mannan from other components was achieved on a Hewlett Packard 5890 Series 2 Gas Chromatograph fitted with a BP 10 capillary column. The BP10 column was 25 M long, had a film thickness of 0.25 µm consisting of 14 % cyanopropylphenyl dimethyl siloxane, and the column internal diameter was 0.2 mm. Helium was used as the carrier gas (1 ml/min), argon as the mixer gas (30 ml/min), the hydrogen had a flow rate of 30 ml/min and the air had a flow rate of 430 ml/min. Peak areas were integrated using a Hewlett Packard 3343 Computing Integrator. Identification of components was achieved using a VG 7070H mass spectrometer equipped with a Finnigan INCOS data system.

The chitin programme consisted of an initial ballistic phase from 50 to 240 °C at 40 °C per min, followed by an 18 min isothermal phase. The mannan analysis program consisted of an initial temperature increase from 50 to 210 °C at 20 °C per min, followed by a 1 °C per min increase to 216 °C, and then a 12 min isothermal phase.

Samples analysed for chitin and mannan content

Primary and secondary phase cultures of *Crinipellis perniciosa* (18 days old) grown as described in 2.1.1b were freeze-dried, ground in a mortar and pestle, and then analysed for both chitin and mannan in order to determine their respective mannan to chitin ratios. Cocoa apical regions (the meristem, up to and including, 4 cm below the apical bud tip) were also freeze-dried, ground in a mortar and pestle, and analysed for the presence of either chitin or mannan, or their corresponding monomers, glucosamine and mannose respectively.

Crab shell chitin, mannan from *Pseudomanas aerogenes*, mannose, galactose, glucose, sedoheptulose, α-D-talose, D-idose, D-gulose, D-altrose, ß-D-allose, D-arabinose, D+glucosamine, D+galactosamine, D+mannosamine, and methylglucamine, were used as standards for co-chromatography and mass spectrometry.

Suppliers

Where appropriate all reagents were glass distilled prior to use. Methylating tubes (sold as culture tubes), Analar HCl, Aristar acetic acid, methanol, chloroform, and ammonium hydroxide (approximately 35g/100 ml) were supplied by BDH Chemicals Ltd. All sugars, amino sugars, internal standards, and trifluoroacetic acid supplied by Sigma Chemical Co. Ltd. Sodium borohydride and acetic anhydride supplied by Lancaster Synthesis Ltd. Magnesium sulphate columns produced in-house. Hydrophilic columns supplied by Waters Ltd. BP 10 capillary column supplied by SGE Ltd. C^{14} labelled D+glucosamine supplied by Amersham Ltd. S. Murray and Co. supplied the 6 ml capacity screw top vials.

3.3.2c Results and Discussion

Chitin assay

The gas chromatogram illustrated in Fig. 3.5 was obtained from primary phase *C. perniciosa* treated in accordance with the new chitin assay. The method produced clean chromatograms with excellent resolution between components. Chitin and methylglucamine (the internal standard) were easily resolved and quantified within 20 min of injection into the GC. The gas chromatogram illustrated in Fig. 3.6 was obtained from apical region cocoa tissue when analysed according to the new chitin assay. The trace illustrated the good resolution obtained with the new procedure in spite of the complexity of the derivatised cocoa tissue. A large number of components were present in the derivatised mixture, but chitin and methylglucamine were still well resolved and quantified within 20 min of injection into the GC.

The results presented in Fig. 3.7 illustrated the different responses of the flame ionisation detector (FID) to the internal standard in relation to glucosamine. The relative response factor for methylglucamine was 1.41 with respect to glucosamine. This relationship exhibited less than 3.5 % variation over the range of ratios 7 to 0.2 of methylglucamine to glucosamine. Such good linearity between different ratios of chitin to methylglucamine ensured reliable quantification was obtained over a range of chitin and hence fungal to plant concentrations.

The mass spectrum illustrated in Fig. 3.8 was representative of those obtained from the chitin of *C. perniciosa*, glucosamine, and crab shell chitin, when treated in accordance with the new chitin assay. Unfortunately, the mass spectra of galactosamine and mannosamine were identical to glucosamine. Therefore, identification using mass spectrometry alone was not possible. In this instance, mass spectrometry was specific only for hexosamines, but this information plus retention time data was sufficient to definitively identify glucosamine, and hence chitin.

Advantages of the new chitin assay

Gas chromatography is currently one of the most sensitive analytical procedures, particularly when a mass spectrometer is used as the detector. A mass spectrometer may be used as a detector with this new methodology in particularly demanding applications. In these experiments when using a flame ionisation detector (FID) the detection limit was about 50 pg of glucosamine per injection. Ultimately, sensitivity depends upon the crystallisation point of each individual sample. An average sample required 50 µl of chloroform per mg of plant material to dissolve the derivatised mixture; the detection limit was therefore about 2.5 ng of chitin/mg sample. The Tsuji *et al.* (1969) colorimetric chitin estimation procedure used by Rocha (1983) and others, had a maximum sensitivity of 6 µg per 25 mg sample (data reworked from Tsuji *et al.*, 1969).

The new method was specific for the hydrolysed product of chitin (glucosamine) rather than being sensitive to a range of six carbon amino sugars. A significant criticism of the colorimetric procedure was that it was not sufficiently specific for glucosamine (Kaminskyj & Heath, 1982; Mayama *et al.*, 1975). The assay was also highly repeatable, the percentage variation between replicates averaged over several experiments was 5.73 % (SD 3.27). This variation was almost entirely due to the extraction step, the variation for all excluding the extraction step was 1.12 % (SD 1.82). The new assay also had a very good percentage recovery. The average percentage recovery was 86.3 % (SD 9.94) for the whole procedure and 91.7 % (SD 7.78) up to the post-acetylation clean up steps. Most of a given sample was therefore lost prior to acetylation. Unfortunately, radiolabelled chitin was unavailable and therefore percentage recoveries could only be calculated for the procedure excluding the extraction step. It was likely therefore, that an additional 10-20 % of a chitin sample was lost during the extraction procedure (Plassard *et al.*, 1982). Therefore, the real percentage recovery for the entire procedure was likely to be about 69-77 %.

The previous, colorimetric based procedures, required about 25 mg of material. The method developed here routinely required [illegible] of material although samples of 200 µg have also

been analysed. Thus, the new method was extremely economical with infected materials, and it would be possible therefore to analyse very specific regions of infected plants.
In addition, the improvement in the sensitivity of the chitin assay ensured that the precision of the mannan chitin ratio determination was correspondingly improved.

Mannan assay

The gas chromatogram illustrated in Fig. 3.9 was obtained from primary phase *C. perniciosa* treated in accordance with the new mannan assay. The method produced clean chromatograms with excellent resolution between components. Mannan and sedoheptulose (the internal standard) were easily resolved and quantified within 20 min of injection into the GC. The gas chromatogram illustrated in Fig. 3.10 was obtained from cocoa apical region tissue treated according to the new mannan assay. The method produced clean chromatograms with good resolution despite the complexity of the derivatised cocoa tissue. A large number of components were present in the derivatised mixture but mannan and sedoheptulose were still well resolved and quantified within 20 min of injection into the GC.

The results presented in Fig. 3.7 illustrated the differing responses of the FID to the internal standard in relation to mannose. The FID relative response factor for sedoheptulose with respect to mannose was 0.714. This relationship was constant over the range of ratios of 5 to 0.2 of sedoheptulose to mannose. Such good linearity between different ratios of mannose to sedoheptulose ensured reliable quantification was achieved over a range of mannan and hence fungal to plant concentrations.

The mass spectrum shown in Fig. 3.11 was representative of those obtained from the mannan of *C. perniciosa*, mannose, and mannan, when treated in accordance with the mannan analysis procedure. Unfortunately, the mass spectra produced by all other hexose sugars were identical to mannose. Therefore, identification using mass spectrometry alone was not possible. In this instance, mass spectrometry was specific for hexose sugars alone, but this information plus retention time data was sufficient to definitively identify mannose, and hence mannan.

Advantages of the new mannan assay

The new mannan assay was probably not significantly more sensitive than the method developed by Jones & Albersheim (1972) and used by Whipps *et al.* (1980 onwards). However, when the method was used in conjunction with a GCMS system, the cleaner samples produced from the procedure ensured unnecessary machine maintenance was minimised. The method of reducing, derivatising, and cleaning the hydrolysates was identical to the improved chitin estimation procedure, and thus the job of estimating mannan to chitin ratios was considerably simplified. The assay was also highly repeatable, the percentage variation averaged over several experiments was 4.68 % (SD 3.5). This variation was almost entirely due to the extraction step, the variation for all excluding the extraction step was 2.00 % (SD 2.53).

The mannan chitin ratios of primary and secondary phase *C. perniciosa*

The results presented in Table 3.1 clearly illustrated the marked difference in the ratio of mannan to chitin (M:C) in the samples of primary and secondary phase *C. perniciosa.* The M:C for the primary phase was 4.53 (SD 0.63) and 2.60 (SD 0.37) in the secondary phase. The relative concentration of mannan equivalents in the secondary phase decreased with respect to the primary phase. The concentration of chitin equivalents remained relatively constant in both growth phases, at 14.83 µg/mg of dry fungal weight (SD 0.77) for the primary and 17.02 µg/mg of dry fungal weight (SD 0.63) for the secondary.

Whipps *et al.* (1980) found a marked difference in the ratio of mannan to chitin between two phases of *Puccinia hordei.* The results from this work indicated a major difference in the mannan chitin ratio between the two growth phases of the *C. perniciosa* life cycle. Therefore, these results were regarded as complementing those of Whipps *et al.* (1980).

There was a relatively small difference in chitin concentrations between the primary and secondary phases of *C. perniciosa.* Thus, a measure of chitin concentration could be used as a measure of relative fungal invasion into cocoa tissue. These results concurred with those working on other plant fungal interactions, as examples; Nandi, 1978; Golubchuk *et al.*, 1960;

Donald & Mirocha, 1977; Lung-chi Wu & Stahman, 1975; Chen & Johnson, 1983; Whipps, 1987; Swift, 1973; Ride & Drysdale, 1971; 1972.

Chitin equivalents in cocoa tissue

The results shown in Table 3.1 indicated chitin constituted about 15 μg/mg of the dry fungal weight of *C. perniciosa* and constituted about 30 ng/mg of dry cocoa tissue. Therefore, a realistic detection limit for chitin in cocoa would be 40 ng/mg of dry sample weight, or equivalent to 0.3 % w/w of dry fungal biomass within a dry cocoa tissue sample. The polymer chitin was probably not present in cocoa tissue but instead almost certainly occurred as glucosamine, the monomeric building block of chitin. The background level of glucosamine detected in cocoa tissue was probably derived from glycoproteins and liberated during acid hydrolysis (Racusen & Foote, 1974).

Rocha (1983) using the Tsuji *et al.* (1969) based colorimetry procedure estimated the chitin concentration in *C. perniciosa* to be about 8.5 μg/mg by weight (reworked from Rocha's data). This figure was somewhat lower than the value of 15 μg/mg obtained here. Rocha (1983) estimated the chitin concentration in cocoa tissue to be about 1 μg/25 mg sample, and this was equivalent to 40 ng/mg of sample. This figure compared very well with the data outlined above. However, for the reasons outlined in 3.1.1, the Rocha (1983) procedure proved to be unsuitable. The previous data obtained in 3.1.1 using the Rocha (1983) procedure indicated the chitin content of cocoa was in the region of 90-120 μg/25 mg of dry cocoa, or 3.6-4.8 μg/mg. Such a high value was the result of a strong background colouration which adversely affected the colorimetry based assay. Rocha (1983) studied cultivars Na32 X UIT1, and IMC67 whereas this work was performed on Amelonado. Therefore, the major point of difference was the level of interfering coloured components which adversely affected the colorimetric procedure for Amelonado, but not apparently for Na32 X UIT1 and IMC67. Thus, the combination of background colouration, interfering chromophores, possible low concentrations of galactosamine, and light scattering from particulate matter defeated the Rocha method, but did not adversely affect the new chitin assay.

Mannan equivalents in cocoa tissue

The data illustrated in Table 3.1 indicated there was a very high background level of mannan in cocoa tissue. The mannan concentration in cocoa constituted about 0.7% dry weight. Because of this, the use of a mannan to chitin ratio estimation procedure may only be applicable in situations where fungal loading constitutes more than about 9% of the total dry weight of the tissue sample.

Mannans are known to occur in hemicellulosic components of cell walls (Preston, 1974). However, other workers using mannan equivalents assays have not found problematically high levels (Whipps *et al.*, 1980; Whipps *et al.*, 1985). Mannans are known to be major components of many gums and mucilages (Preston, 1974), for example, the galactomannans. Thus, it was possible that the major source of mannan in cocoa tissue may be derived from mucilage, of which, cocoa is known to be a prolific producer.

The difference in the mannan chitin ratio between the primary and secondary phases was probably insufficient to allow the apportioning of the two phases in infected cocoa. A detailed mathematical investigation would need to be performed to see whether it was possible. In the light of these results it was unnecessary to do this investigation because the procedure could not realistically be used in cocoa tissue.

3.3.2d Conclusions

All the data indicated a new, more sensitive, and specific chitin assay had been developed. In addition, the data indicated a new and improved mannan assay had also been developed. There was a significant difference in the mannan chitin ratio between the primary and secondary phases of *C. perniciosa*. The ratios for the primary and secondary phases were 4.53 (SD 0.63) and 2.61 (SD 0.37) respectively. The concentration of mannan was lower in the secondary phase of *C. perniciosa*. There appeared to be no significant difference in the chitin concentration between the primary and secondary phases of growth.

Cocoa (*Theobroma cacao*) contained a very high level of mannan and a background level of chitin, probably as glucosamine. Because of this, the use of a mannan to chitin ratio estimation procedure may only be applicable in situations where fungal loading constituted more than about 9% of the total dry weight of the tissue sample. However, a chitin assay may be used if the concentration exceeds 0.3% w/w of cocoa tissue.

Fig. 3.1. Standard curve for a range of glucosamine concentrations in uninfected cocoa tissue.

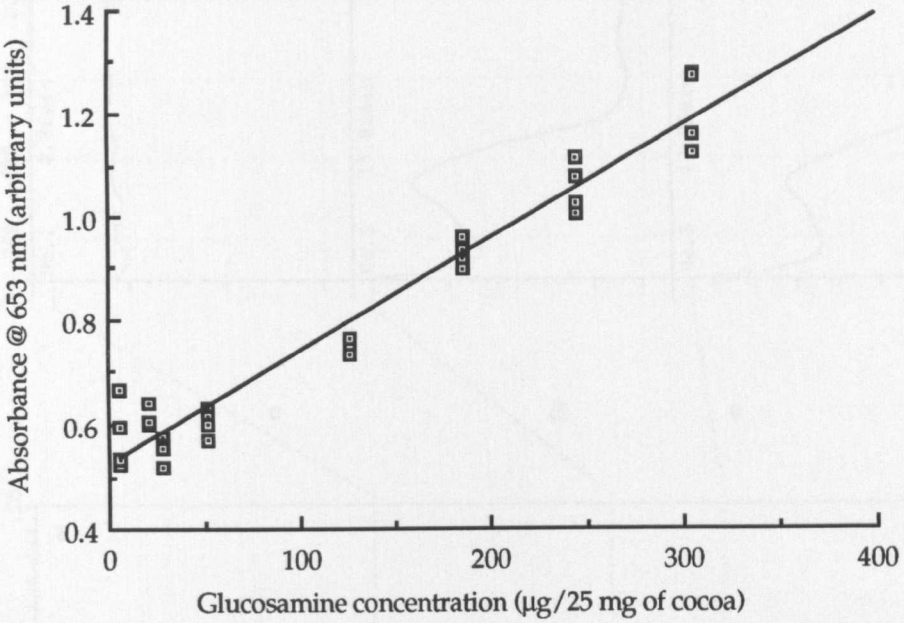


Fig. 3.2. High performance liquid chromatogram (a) of mannose and glucosamine using the Oshima and Kumanotami (1983) derivatisation procedure. Online spectra (b) of individual peaks are as follows; solvent (c), mannose (d), and glucosamine (e).

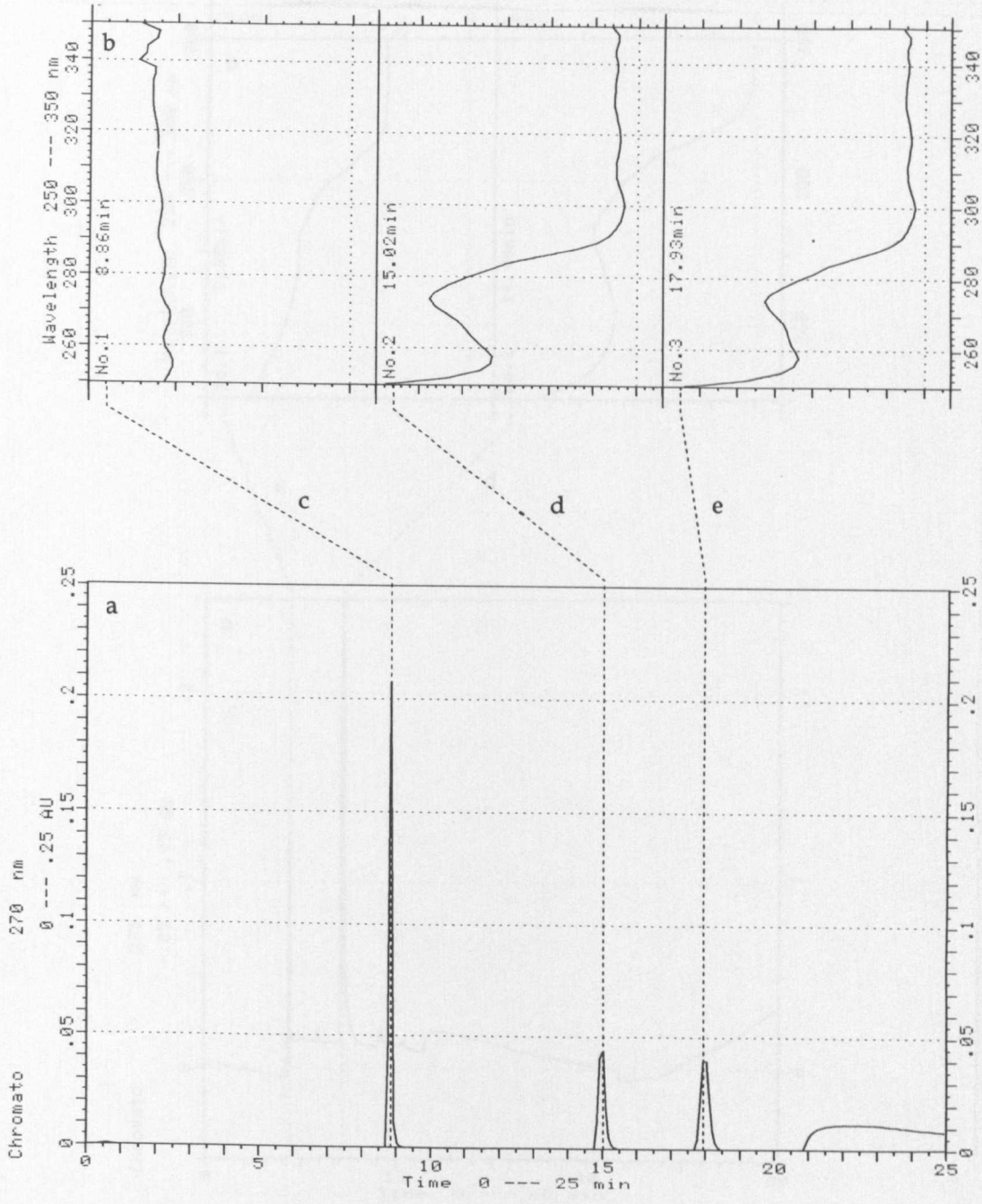


Fig. 3.3. High performance liquid chromatogram (a) of glucose using the Oshima and Kumanotami (1983) derivatisation procedure. Online spectra (b) of individual peaks are as follows; solvent (c), and glucose (d).

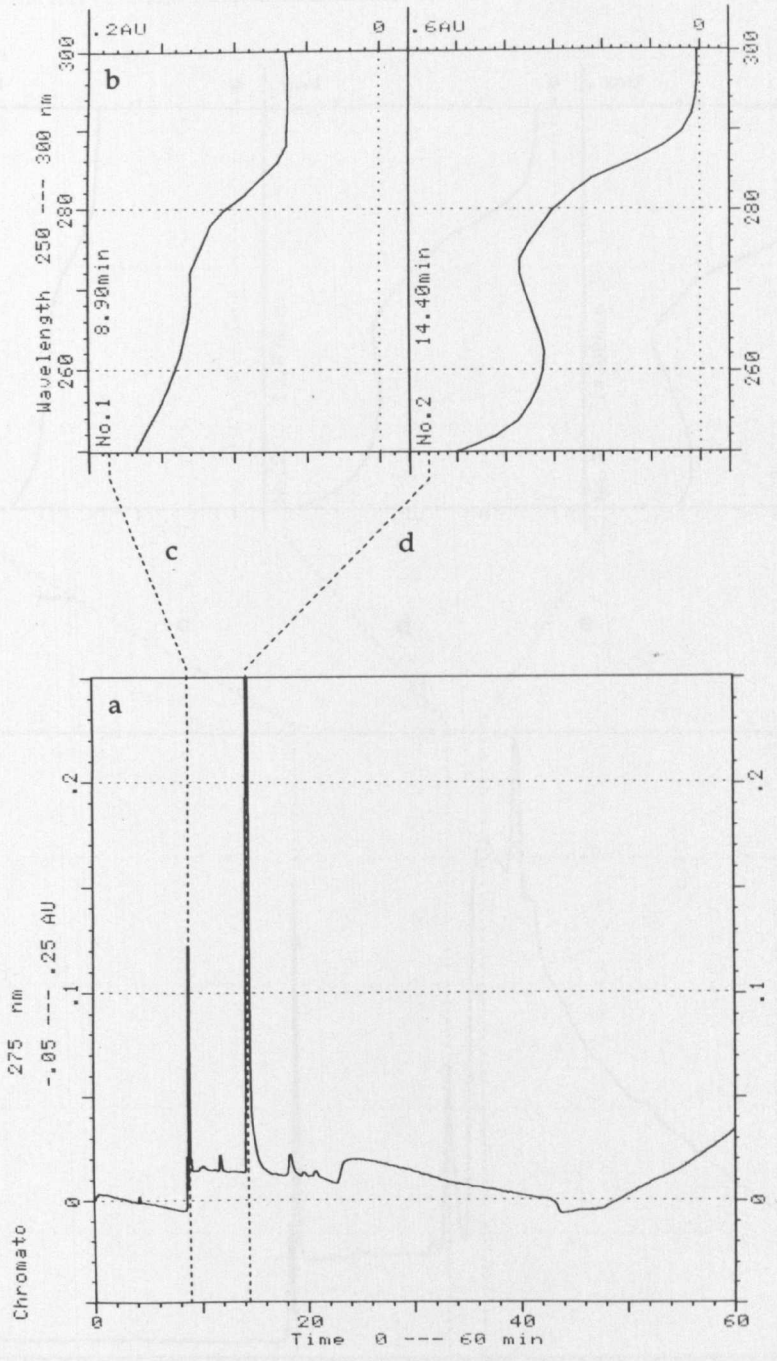


Fig. 3.4. High performance liquid chromatogram (a) of cocoa tissue extracted as described in the main text and using the Oshima and Kumanotami (1983) derivatisation procedure. Online spectra (b) of individual peaks are as follows; solvent (c), unknown hexose component (d) and glucose (e).

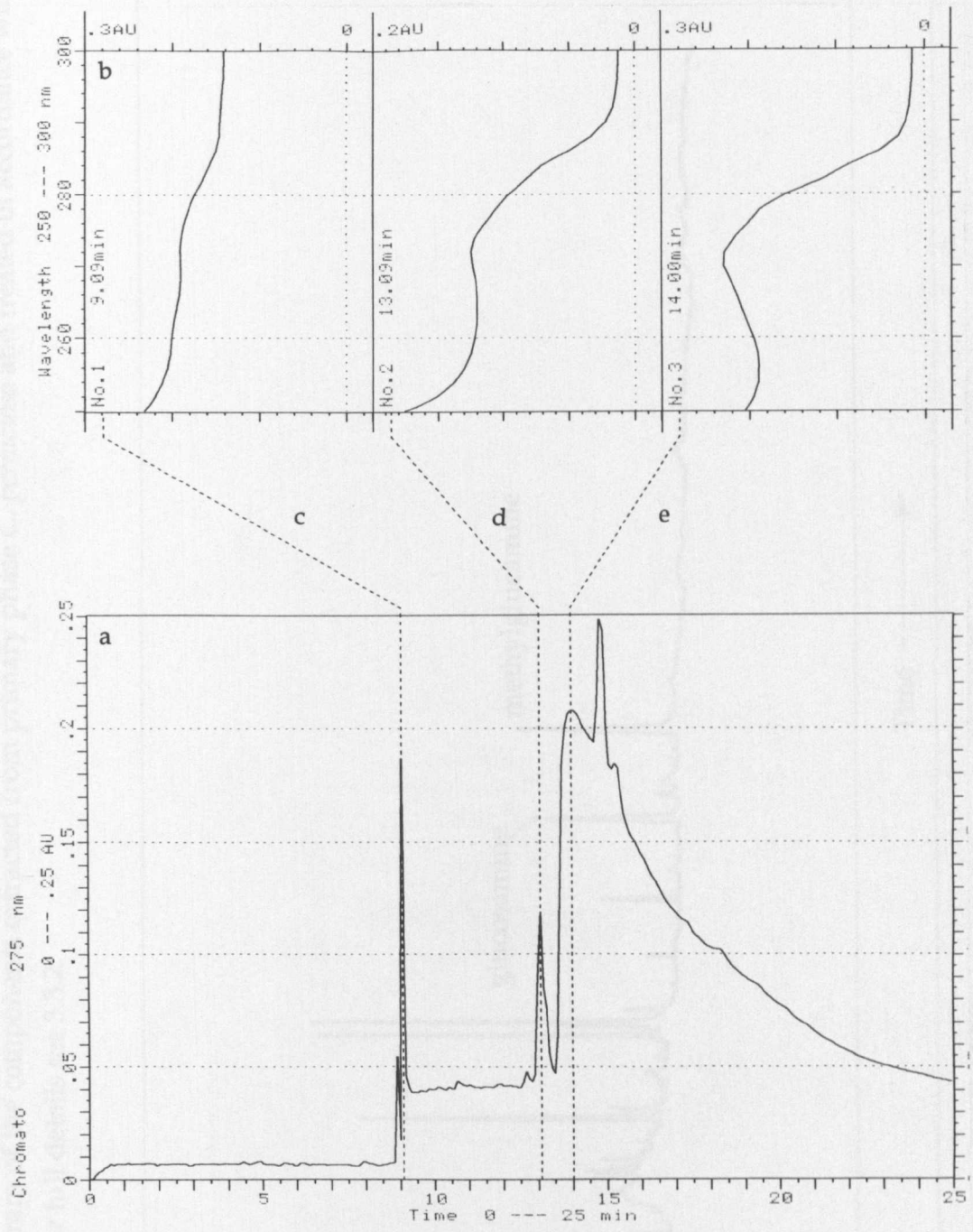


Fig. 3.5. Gas chromatogram of the components extracted from primary phase *C. perniciosa* and treated in accordance with the new chitin assay. For full details see 3.3.2.

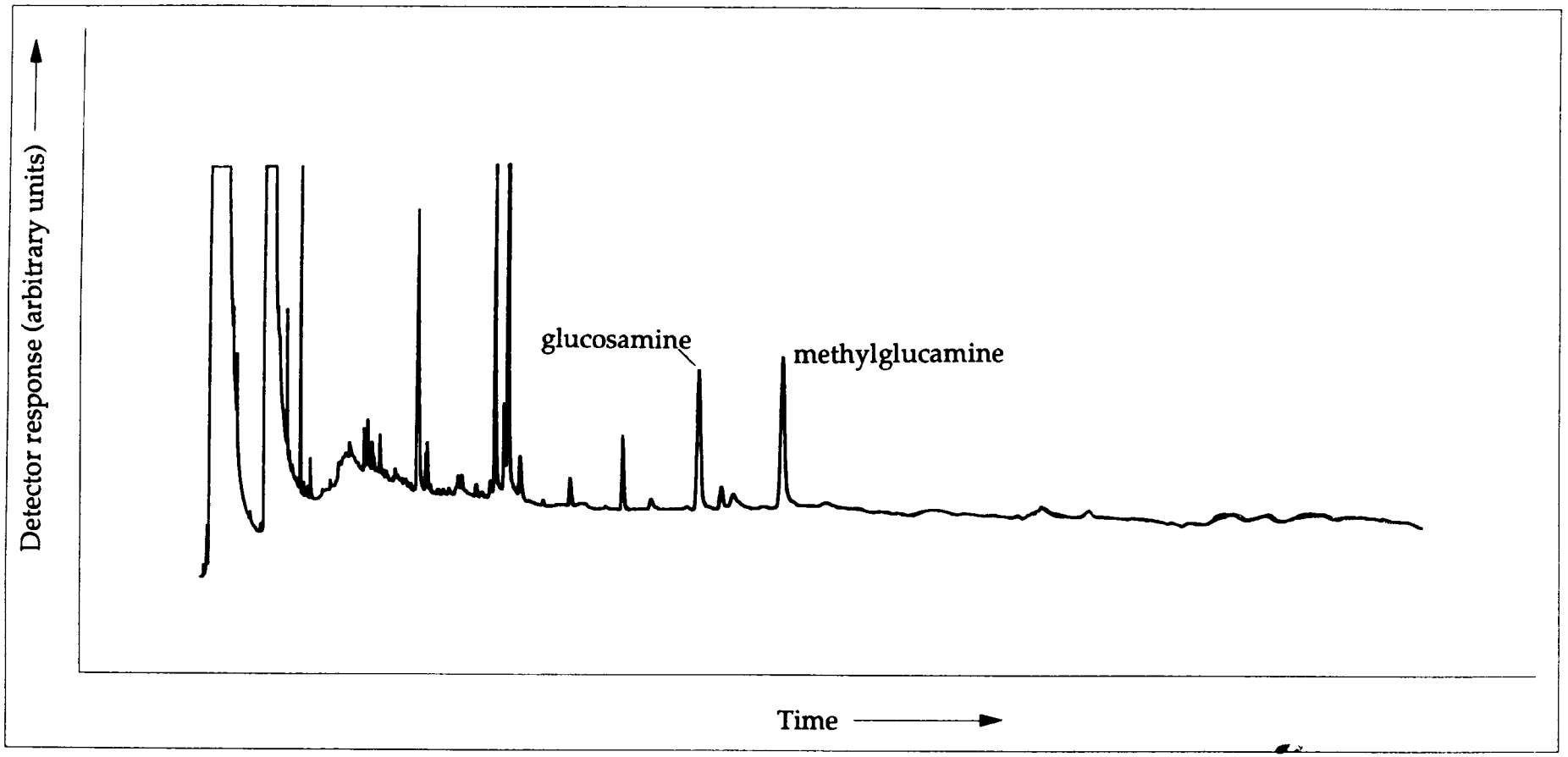


Fig. 3.6. Gas chromatogram of the components extracted from cocoa apical region tissue and treated in accordance with the new chitin assay. For full details see 3.3.2.

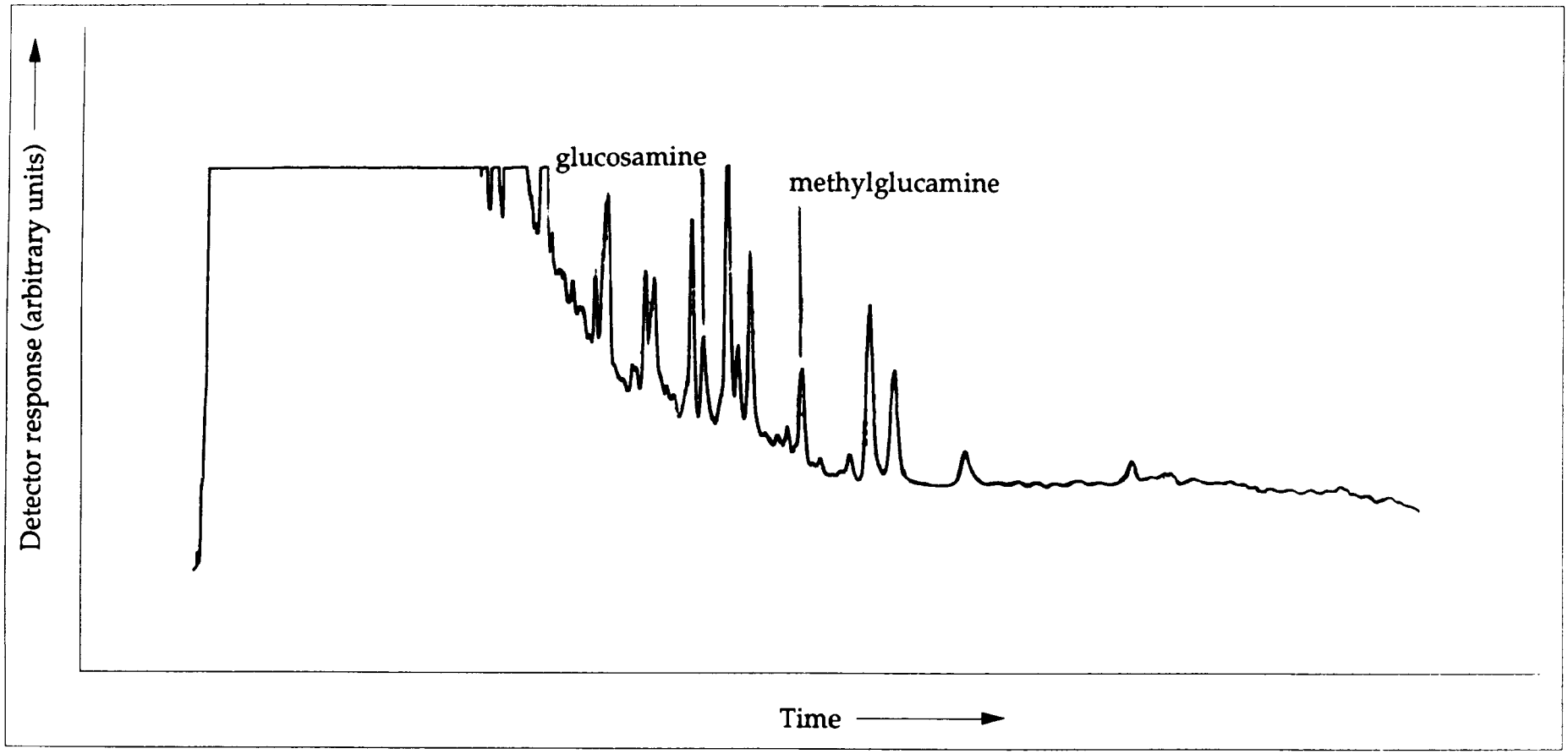


Fig. 3.7. Flame ionisation detector relative response factors for a range of ratios of the respective internal standard to either glucosamine or mannose (maximum variation 6.2%).

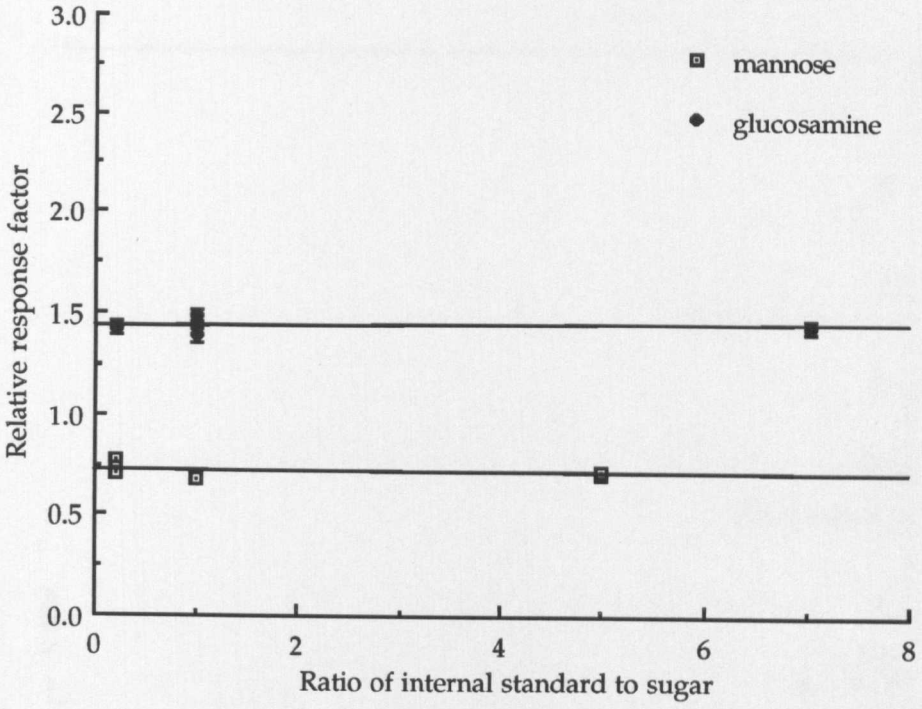


Fig. 3.8. Mass spectrum of chitin when treated in accordance with the new chitin assay. For full details see 3.3.2.

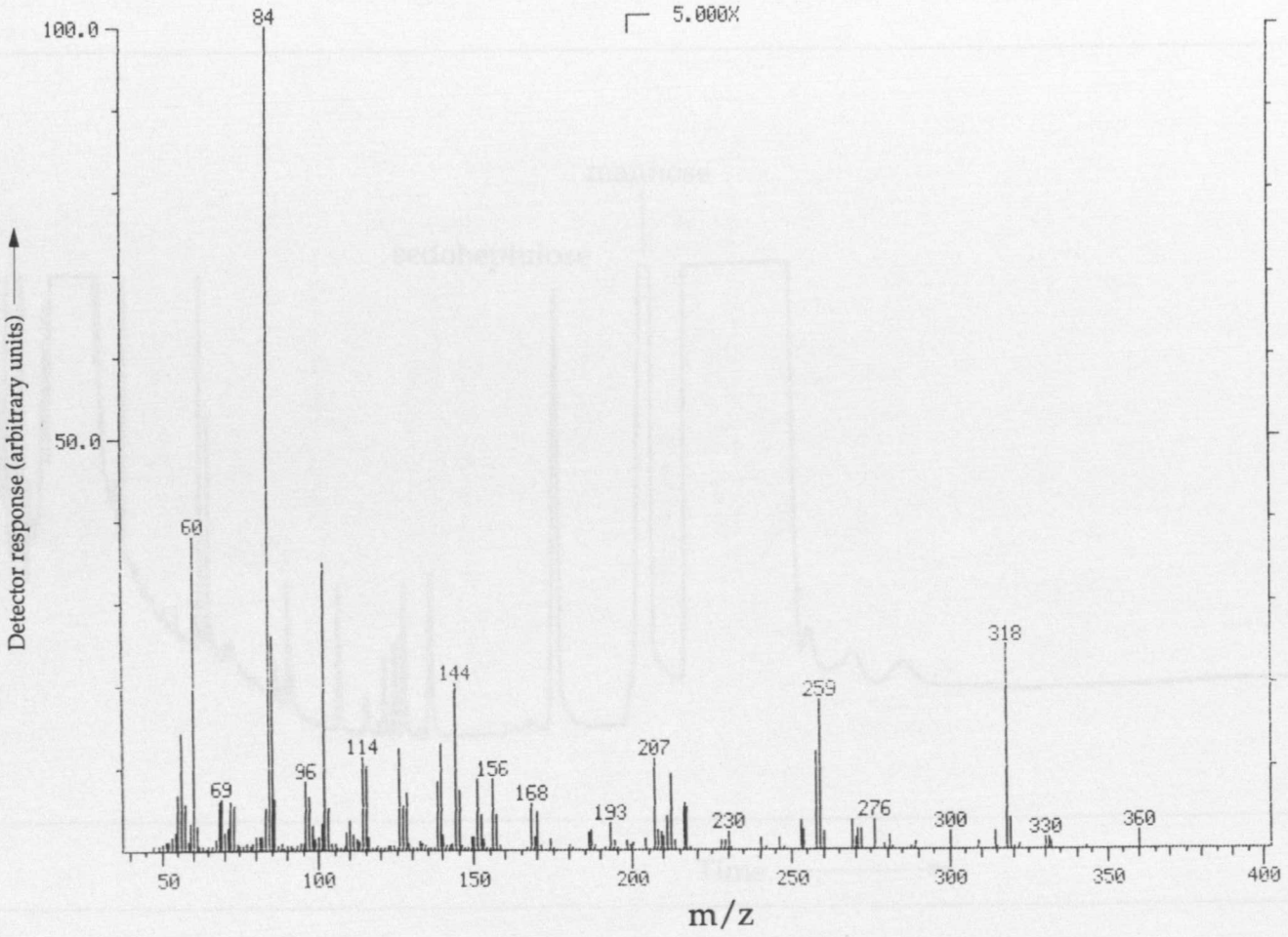


Fig. 3.9. Gas chromatogram of the components extracted from primary phase *C. perniciosa* and treated in accordance with the new mannan assay. For full details see 3.3.2.

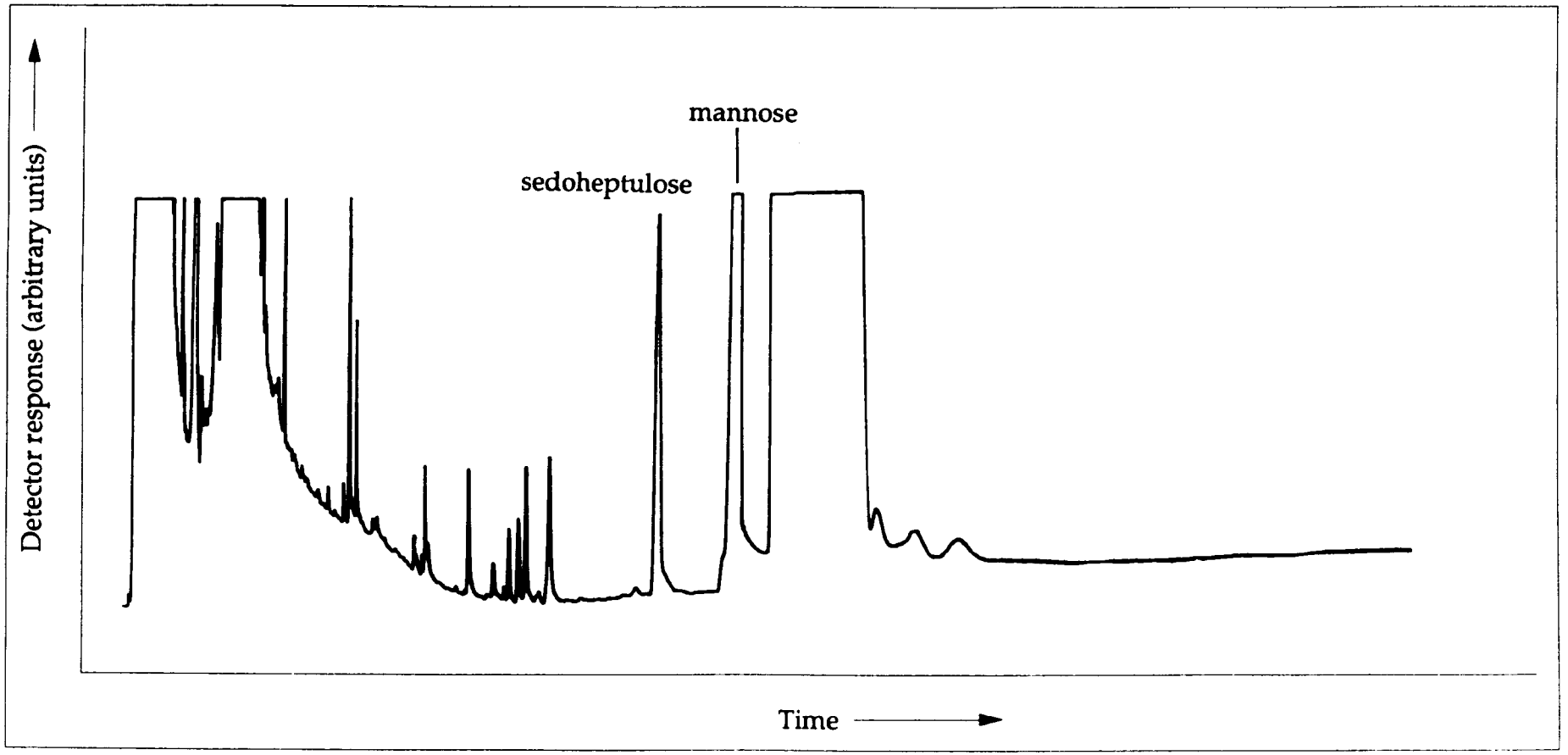


Fig. 3.10. Gas chromatogram of the components extracted from cocoa apical region tissue and treated in accordance with the new mannan assay. For full details see 3.3.2.

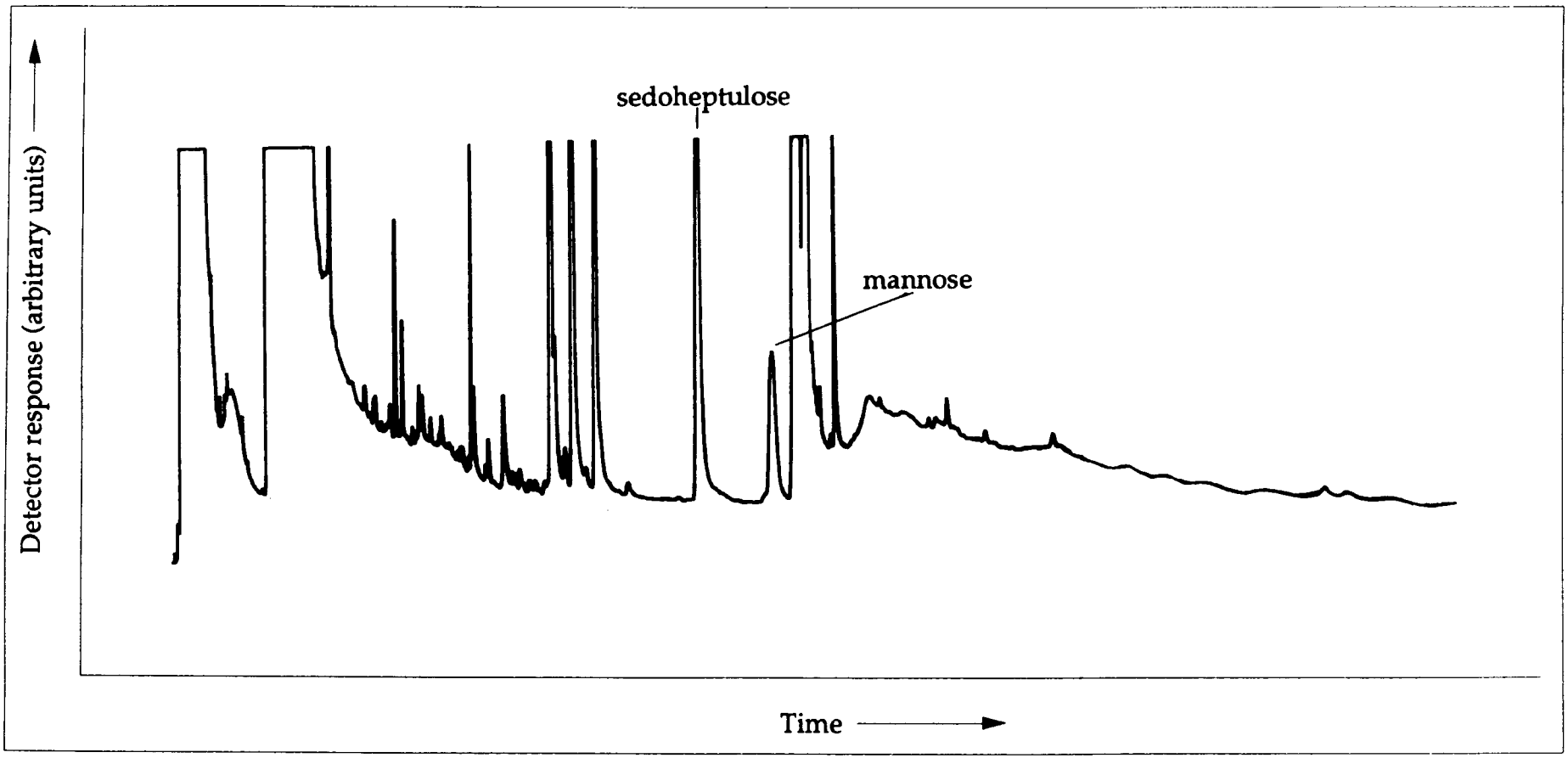


Fig. 3.11. Mass spectrum of mannan when treated in accordance with the new mannan assay. For full details see 3.3.2.

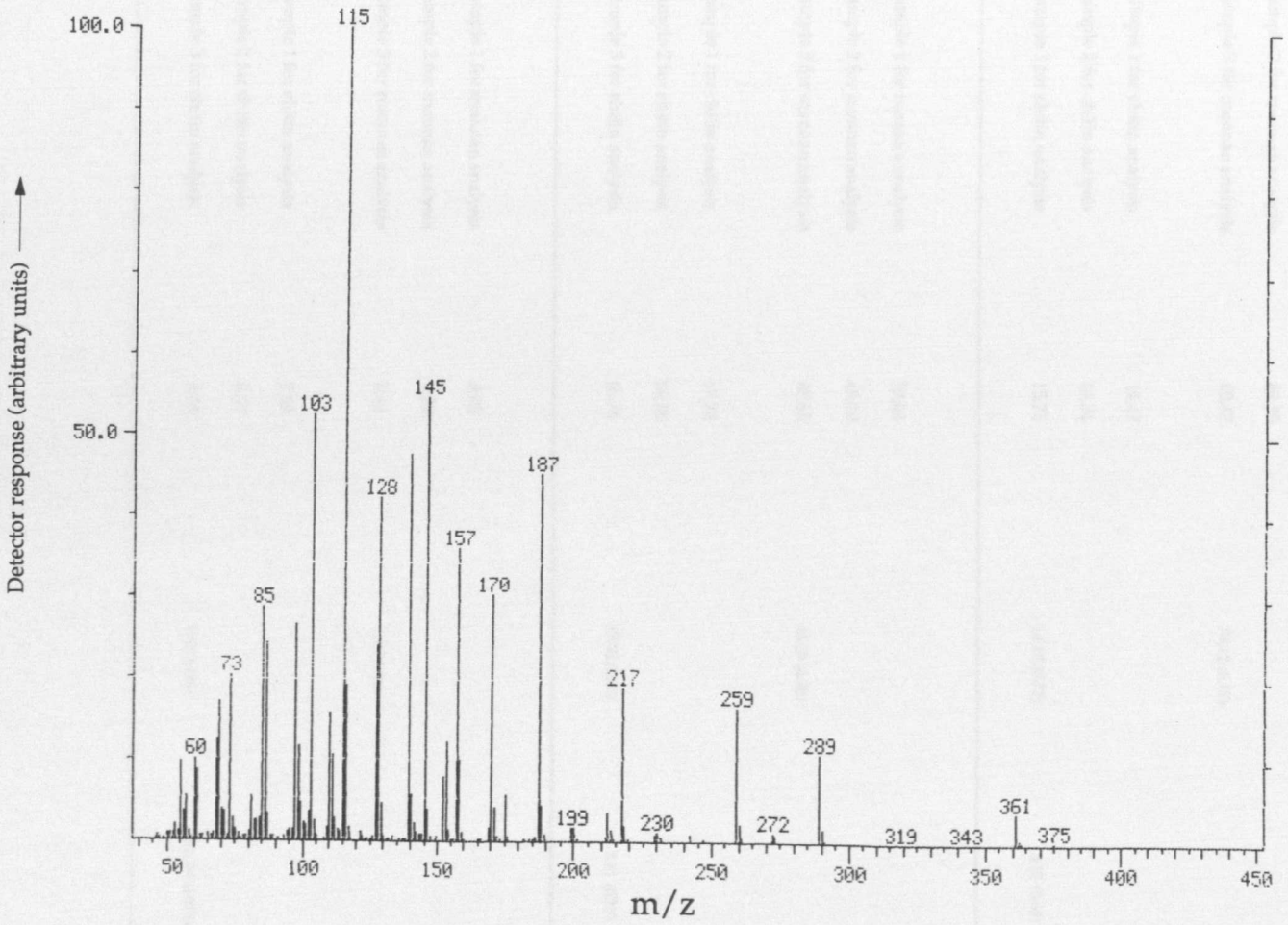


Table 3.1. The concentrations and ratios of chitin and mannan in primary and secondary phase *C. perniciosa* and cocoa apical region tissue.

	Description	Concentration of chitin or mannan µg/mg of tissue	Mean concentration µg/mg (standard deviation)	Mannan chitin ratio (standard deviation)
	Sample 1 for mannan analysis	71.64		
	Sample 2 for mannan analysis	69.59		
	Sample 3 for mannan analysis	60.12	76.12 (6.15)	
Primary phase				
	Sample 1 for chitin analysis	14.47		
	Sample 2 for chitin analysis	14.31		
	Sample 3 for chitin analysis	15.71	14.18 (0.77)	4.55 (0.63)
	Sample 1 for mannan analysis	39.05		
	Sample 2 for mannan analysis	45.20		
	Sample 3 for mannan analysis	48.63	44.29 (4.85)	
Secondary phase				
	Sample 1 for chitin analysis	17.74		
	Sample 2 for chitin analysis	16.58		
	Sample 3 for chitin analysis	16.74	17.02 (0.63)	2.61 (0.37)
	Sample 1 for mannan analysis	8.95		
	Sample 2 for mannan analysis	7.93		
	Sample 3 for mannan analysis	6.44	7.77 (1.26)	
Cocoa				
	Sample 1 for chitin analysis	0.04		
	Sample 2 for chitin analysis	0.02		
	Sample 3 for chitin analysis	0.04	0.03 (0.01)	282. 09 (143.16)

Chapter 4

The neutral and amino sugar components of primary and secondary phase C. *perniciosa*

4.1.1a Introduction

The results from the previous chapter indicated the ratio of mannan to chitin was significantly different in the primary and secondary growth phases of *C. perniciosa*. Mannan is a hexose (neutral) sugar polymer and chitin is a hexosamine (amino) sugar polymer. It was therefore decided to broaden the scope of this previous work to encompass other neutral and amino sugars, by harnessing the newly developed, highly sensitive and specific chitin assay, and by using the new mannan assay.

The following work was designed to determine the neutral and amino sugar profiles of the primary and secondary growth phases of *C. perniciosa*, as well as cocoa apical region tissues. This allowed the identification of any potentially useful ratios between sugar components. The work was also designed to identify neutral and amino sugars unique to *C. perniciosa*, and those specific to either growth phase.

Potential problems associated with neutral and amino sugar analysis

There is only one potential problem associated with neutral and amino sugar analysis procedures. Individual neutral and amino sugar contents estimated *in vitro* may not adequately reflect the true concentration when the fungus grows within the host plant. Thus, any fungal or fungal growth phase estimation procedure based on the neutral or amino sugar content measured *in vitro* may not adequately reflect true fungal loading. However, this is unlikely to prove a problem as the relative distribution of the fungus or fungal growth phase in the host plant could still be reliably estimated.

4.1.1b Methods

Amino sugar analysis

The methods for preparing the acetylated derivatives for amino sugar analysis were identical to those used for the chitin assay described in 3.3.2b. For the remainder of this chapter the chitin assay will be referred to as the amino sugar assay.

Neutral sugar assay

The methods for preparing the acetylated derivatives for neutral sugar analysis were identical to those of the new mannan assay described in 3.3.2b. For the remainder of this chapter the mannan assay will be referred to as the neutral sugar assay.

Gas chromatography

Separation, quantification, and identification using mass spectrometry of amino and neutral sugars was achieved using the same equipment and experimental conditions as those previously used for the newly developed chitin assay and the new mannan assay respectively.

Each sample was run twice on the GC. For the first run, the samples were diluted with 50 µl of chloroform per mg of sample. This allowed the separation and quantification of low concentration components. For the second, the samples were diluted with 250 µl of chloroform per mg of sample. This allowed the separation and quantification of the higher concentration components. It was not possible to analyse all components at once because of chromatographic limitations, such as column overloading.

Care was taken when interpreting the non-hexose or non-hexosamine data as in many cases the components may have differed chemically from the internal standards. Generally, to obtain a reliable estimate for the concentration of a specific component the internal standard would have to be chemically very similar to the component under study. Thus, the internal standards were not used to obtain definitive estimates of the concentrations of non-hexose or non-hexosamine components; but, they were used to estimate the relative concentrations across different samples. The aim of this study was to screen *C. perniciosa* tissues to elucidate the relative concentrations of neutral and amino sugar components in the primary and secondary phases of growth. The aim was to highlight the differences, not to provide absolute concentrations of non-hexose or -hexosamine components. When relative and not absolute non-hexose or -hexosamine component concentrations were required the method was adequate and the results accurate. The concentrations of all components were therefore

given in arbitrary units (Au). One Au was roughly equivalent to 1 mg and 1 μAu was equivalent to 1 μg.

Samples analysed

Primary and secondary phase cultures of *Crinipellis perniciosa* (18 days old) grown as described in 2.1.1b were analysed for both neutral and amino sugars. Cocoa apical regions (the meristem, up to and including, 4 cm below the apical bud tip) of the cultivar Amelonado were also screened for the presence of neutral and amino sugars. Mannose, galactose, glucose, sedoheptulose, α-D-talose, D-idose, D-gulose, D-altrose, β-D-allose, D-arabinose, D+glucosamine, D+galactosamine, D+mannosamine, and methylglucamine were used as standards for co-chromatography and mass spectrometry.

Statistical treatment of results

The central aim of the following analysis procedure was to highlight any components that may have potentially formed part of a useful ratio. This was done firstly, by identifying the components found in significantly different concentrations in the two phases of *C. perniciosa*. And secondly, by identifying any components that exhibited little variation between life cycle phases and could therefore serve as a level from which other components could vary. The procedure was also designed to detect components specific to either the primary or secondary phases, or any that could be used as biomarkers for *C. perniciosa*. The procedure was as follows:-

Step 1

Components isolated using either the neutral or amino sugar analysis procedures with concentrations more than 1 μAu/mg of dry fungal weight were highlighted on representative chromatograms obtained from samples of primary and secondary phase *C. perniciosa*. These peaks were then located and the components quantified on two other chromatograms obtained from samples of primary and secondary phase *C. perniciosa*. The component concentrations were then assessed for significant differences in concentration between the primary and secondary phases, using a one way analysis of variance (ANOVA). The Null

hypothesis was: the concentration of the component is identical in both growth phases of *C. perniciosa*. Thus, components which exhibited relatively large or random variations were excluded from the ratio analysis procedure. The components rejected by the ANOVA were treated in accordance with Step 3.

Step 2

The components not rejected by the previous ANOVA were then assessed to determine whether their concentrations varied significantly around a grand mean encompassing both growth phases. If the variation was small then they may have served as a base from which other components could vary and thus potentially may have formed part of a useful ratio. The percentage variation was calculated for each component representing more than 1 μAu/mg of dry fungal weight on each reference chromatogram described above. A variation of 33 % or less from the overall mean encompassing both growth phases was considered acceptable. This level of variation was considered to be at the outer limits of acceptability and would be certain not to miss any potentially useful components. Components which occurred exclusively in only one phase were then analysed in accordance with Step 5. Thus components with large or random variations in concentration were eliminated from the statistical analysis procedure.

Step 3

Ratios were calculated for all possible combinations of components highlighted in Steps 1 and 2. The resulting ratios were then assessed for significance using a one way ANOVA. The null hypothesis was: the ratio between the two components is identical in both growth phases of *C. perniciosa*. Ratios rejected by the ANOVA were considered to be potentially useful.

Step 4

The chromatograms produced from the cocoa extracts were then screened for the presence of either of the components which formed the potentially useful ratios.

Step 5

Components which occurred at more than 1 μAu/mg of dry fungal weight and found in only the primary or secondary phases of growth were assessed for phase specific biomarker potential. Thus potentially, they could be used to estimate the concentration of either the primary or secondary phase in a mixture of the two. A variation of less than 33 % for all samples from the overall phase mean was considered acceptable. Cocoa tissue was then screened for the presence of the potential phase marker.

Step 6

Components which occurred at more than 1 μAu/mg of dry fungal weight were assessed for fungal biomarker potential, i.e. could be used to estimate the *C. perniciosa* biomass in a cocoa sample. A variation of 33 % or less from the overall mean encompassing both growth phases was considered acceptable. This level of variation was considered generous and was certain not to miss any possibilities. Chromatograms obtained from the extracts of cocoa tissue were then screened for the presence of the potential biomarkers.

Step 7

The same reference samples described in Step 1 were then run on a mass spectrometer, as described in 3.3.2b. Mass spectra were obtained for all possible peaks. The mass spectra obtained from the peaks in our samples were compared with those in the inbuilt mass spectrometer library which contained more than 30,000 spectra. For each potential match the computer calculated a score out of 1000. Eight hundred or above was considered sufficient to identify a compound. Therefore, all peaks registering a score of 800 or more were considered to have been identified. In addition, because the mass spectra for the hexoses were identical then retention time data was also used to aid in the identification. The hexosamines were treated in the same way.

Cocoa was not screened first for the presence of the components for two reasons. Firstly, the approach needed to be validated in order to see if it could be used for other classes of

compounds. And secondly, the procedure may have highlighted ratios that other workers might have found useful.

4.1.1c Results and Discussion

The chromatogram shown in Fig. 4.1 was obtained from primary phase *C. perniciosa* treated in accordance with the neutral sugar analysis procedure. The seventeen components which occurred at a concentration of 1 μAu/mg of dry fungal weight or greater were indicated by the lower case letters 'a'-'q' inclusive. The results show the excellent component resolution obtained with the new procedure despite the complexity of the derivatised extract. The chromatogram shown in Fig. 4.2 was obtained from primary phase *C. perniciosa* treated in accordance with the amino sugar extraction procedure. The fourteen components which occurred at a concentration of 1 μAu/mg of dry fungal weight or greater were highlighted by the lower case letters 'aa'-'an' inclusive. The results once again demonstrated the excellent component resolution obtained with the new amino sugar analysis procedure.

The chromatogram shown in Fig. 4.3 was obtained from cocoa apical region tissue treated in accordance with the neutral sugar extraction procedure. The results demonstrated the excellent resolution between components achieved using the neutral sugar analysis procedure despite the complexity of the derivatised extracts. The chromatogram illustrated in Fig. 4.4 was obtained from cocoa apical region tissue treated in accordance with the amino sugar analysis procedure. The results demonstrated the good resolution obtained between components, despite the large number of components present in the complex mixture.

The data presented in Fig. 4.5 and 4.6 illustrated the relative concentrations of the 31 different sugar components in the primary and secondary phases of the fungus. Please note, log scales were used on the graphs and therefore error bars could not be plotted accurately. Therefore, standard deviations were shown on the top of each bar.

The data presented in Table 4.1 summarised those components with significant concentration differences in either the primary or secondary phases of the fungus, a total of ten were

identified. The data presented in Table 4.2 lists the ratios that differed significantly between the primary and secondary phases of the fungus, a total of ten were identified. The component identifications shown in Table 4.3 were obtained using the built-in data analysis system on the mass spectrometer, and also with the aid of retention time data.

Steps 1 and 2

Seventeen components extracted by the neutral sugar procedure were highlighted as occurring in either growth phase of *C. perniciosa* at a concentration of 1 μAu/mg of dry fungal weight or greater (see Fig. 4.5). Fourteen components extracted by the amino sugar procedure were found to occur at a concentration of 1 μAu/mg of dry fungal weight or greater (see Fig. 4.6).

Three components extracted by the neutral sugar procedure were identified by the ANOVA as occurring in significantly different concentrations (P>= 0.1) in either growth phase of the fungus. These components were 'b', 'c', and 'q' (see Table 4.1). Seven components extracted by the amino sugar procedure were identified by the ANOVA as being in significantly different (P>= 0.1) concentrations in either growth phase of the fungus. These components were 'aa', ac', 'ad', 'af', 'ag', 'ak', and 'al' (see Table 4.1). No components failed the first ANOVA (Step 1) and varied by less than 33 % around their grand mean. Thus, 10 components were carried through to the next analysis step.

Of these 10 components, 'b', 'c', 'ak', and 'al' appeared to be specific to the secondary phase. These components were statistically analysed in greater detail below (Step 5). The remaining six components, 'q', 'aa', 'ac', 'ad', 'af', and 'ag', were evaluated using ratio analysis.

Step 3 and 4

In all, ten potentially useful ratios were highlighted by the procedure (see Table 4.2). Unfortunately, all of the ratios contained components which occurred in cocoa tissue; and therefore they could not be used for estimating the relative proportions of the primary and secondary growth phases of *C. perniciosa* in infected cocoa.

Strangely, the statistical analysis procedure did not highlight the ratio of mannan to chitin as being different between the two growth phases. On closer inspection, from these later results it was clear that there was very little difference in the mannan chitin ratio. The ratio for the primary phase was 4.78 (SD 0.81) and 4.16 (SD 0.39) for the secondary. These results contradicted our earlier findings (3.3.2c). Previously, the mannan chitin ratio was estimated for the primary phase as 4.53 (SD 0.63) and 2.6 (SD 0.37) for the secondary phase. The samples were grown and processed under identical conditions, but the results differed markedly. However disappointing the results appear, for all practical purposes the mannan chitin ratio estimation procedure could not be used in cocoa because of the high concentration of mannan equivalents in cocoa tissue. Therefore the contradiction in results was not further investigated.

Step 5

Components 'b', 'c', 'ak', and 'al' which appeared to be specific to the secondary phase were then investigated. The concentration of components 'ak' and 'al' varied by more than 33 % and their average concentration was less than 1 μAu/mg of dry fungal weight. Thus, they were of no value for the purposes of this project. The remaining components, 'b' and 'c', exhibited less than the required 33 % variation (31% SD 9.5 and 22.9 % SD 12.33 respectively). Therefore, these components had potential as phase specific markers. Unfortunately, 'c' occurred at a very low average concentration of 0.79 μAu/mg of dry fungal weight (SD 0.18). Therefore 'c' was of no value. Component 'b' could not be used because it occurred in significant concentrations in cocoa tissue. Component 'b' was in fact galactose (see Table 4.3).

It was hoped that a useful phase specific marker would be discovered. Such a marker may have allowed the relative proportions of the two phases to be calculated. However, the results indicated there were basic biochemical differences between the phases and these underscored the physiological differences. It was unfortunate that components 'c', 'ak', and 'al' occurred in very low levels and that the latter two occurred in very variable concentrations, but the important point was that differences were found. Component 'b' would have been an ideal phase specific marker because it occurred in relatively high

concentrations in the secondary phase, 8.64 μg/mg of dry fungal weight (SD 2.36), exhibited acceptable levels of variation, and was absent from the primary phase. But unfortunately, it occurred in cocoa tissue. The one surprising feature was that it did not occur in the primary phase, galactose is a common if not universal constituent of numerous organisms.

The 31 components were then assessed for biomarker potential. At first glance it may seem that this was a repeat of Step 2. But, component concentrations could differ between the phases (were rejected by the ANOVA) and the difference between phases may still have been less than 33 %. This proved to be true. Two components found to have significant differences in concentration between the different growth phases were highlighted by the procedure as being potentially useful biomarkers. These components were 'q' and 'aa', which were mannose and glucosamine respectively. The remaining 29 components were rejected because they either occurred in too low a concentration in the fungus with respect to their level in cocoa, were present on average at less than 1 μAu/mg of dry fungal weight, or varied by more than 33 % from their respective overall mean encompassing both growth phases.

Unfortunately, earlier work (3.3.2c) demonstrated that mannose was present in cocoa in significant concentrations (7.77 μg/mg of dry cocoa weight SD 1.26) and could not therefore be used as a fungal specific biomarker. However, glucosamine was a viable biomarker for C. *perniciosa* in cocoa tissue. Earlier work (3.3.2c) demonstrated that it was present in relatively low concentrations in cocoa tissue (0.03 μg/mg of dry cocoa weight SD 0.01) and the work in this chapter indicated that it was present in *Crinipellis* in reasonable concentrations (12.42 μg/mg of dry fungal weight SD 2.18). The results also demonstrated that it exhibited less than 33 % variation between phases. Thus, a viable biomarker for estimating fungal loading in cocoa tissue infected with C. *perniciosa* was identified. These results concurred with Whipps *et al.* (1980) who found that chitin and mannan were valuable biomarkers for fungal loading. It was unfortunate in this instance that mannan estimation could not be used in infected cocoa tissue. These results also concurred with those working on other plant/fungal interactions, as examples; Nandi, 1978; Golubchuk *et al.*, 1960; Donald & Mirocha, 1977; Lung-

chi Wu & Stahman, 1975; Chen & Johnson, 1983; Whipps, 1987; Swift, 1973; Ride & Drysdale, 1971; 1972.

Step 7

Using the mass spectrometer, 7 components were identified in the primary and secondary phases of the fungus, and a further 3 components in cocoa tissue (see Table 4.3).

4.1.1d Conclusions

All of the components extracted and analysed by the neutral and amino sugar analysis procedures were quantified in the primary and secondary growth phases of *C. perniciosa*. Thirty one components extracted by both sugar analysis procedures were highlighted as occurring in either growth phase at a concentration of more than 1 μAu/mg of dry fungal weight. Ten components occurred in significantly different concentrations in either growth phase and could therefore be used for ratio analysis. Ten ratios were highlighted that would have allowed, in principle, the quantification of the relative concentrations of primary and secondary phase *C. perniciosa* in infected cocoa tissue. Unfortunately, all ratios contained components that occurred in significant concentrations in cocoa with respect to the concentration in the fungus.

A mannan chitin ratio was not among the 10 ratios highlighted as being significantly different between the primary and secondary phases of *C. perniciosa*. This contradicted the results from 3.3.2c. However, this was not a further problem because mannan equivalents occurred in cocoa at relatively high concentrations, and thus the mannan chitin ratio was of no use for the estimation of the relative proportions of primary and secondary phase mycelia in infected cocoa tissue.

Four components appeared to be specific to the secondary phase of *C. perniciosa*. Unfortunately, these components either exhibited highly significant variation or occurred in too low a concentration to be useful as secondary phase specific biomarkers.

All 31 components were assessed for *C. perniciosa* biomarker potential. Thirty were unsuitable because they occurred at too low a concentration in the fungus relative to cocoa, or they exhibited highly significant variation between phases. However, the results confirmed definitively that the remaining component, chitin, was a valuable *C. perniciosa* specific biomarker.

Fig. 4.1. Gas chromatogram of the components extracted from primary phase *C. perniciosa* and treated in accordance with the neutral sugar analysis procedure. For full details see 4.1.1. Peak labels are those referred to in the main text.

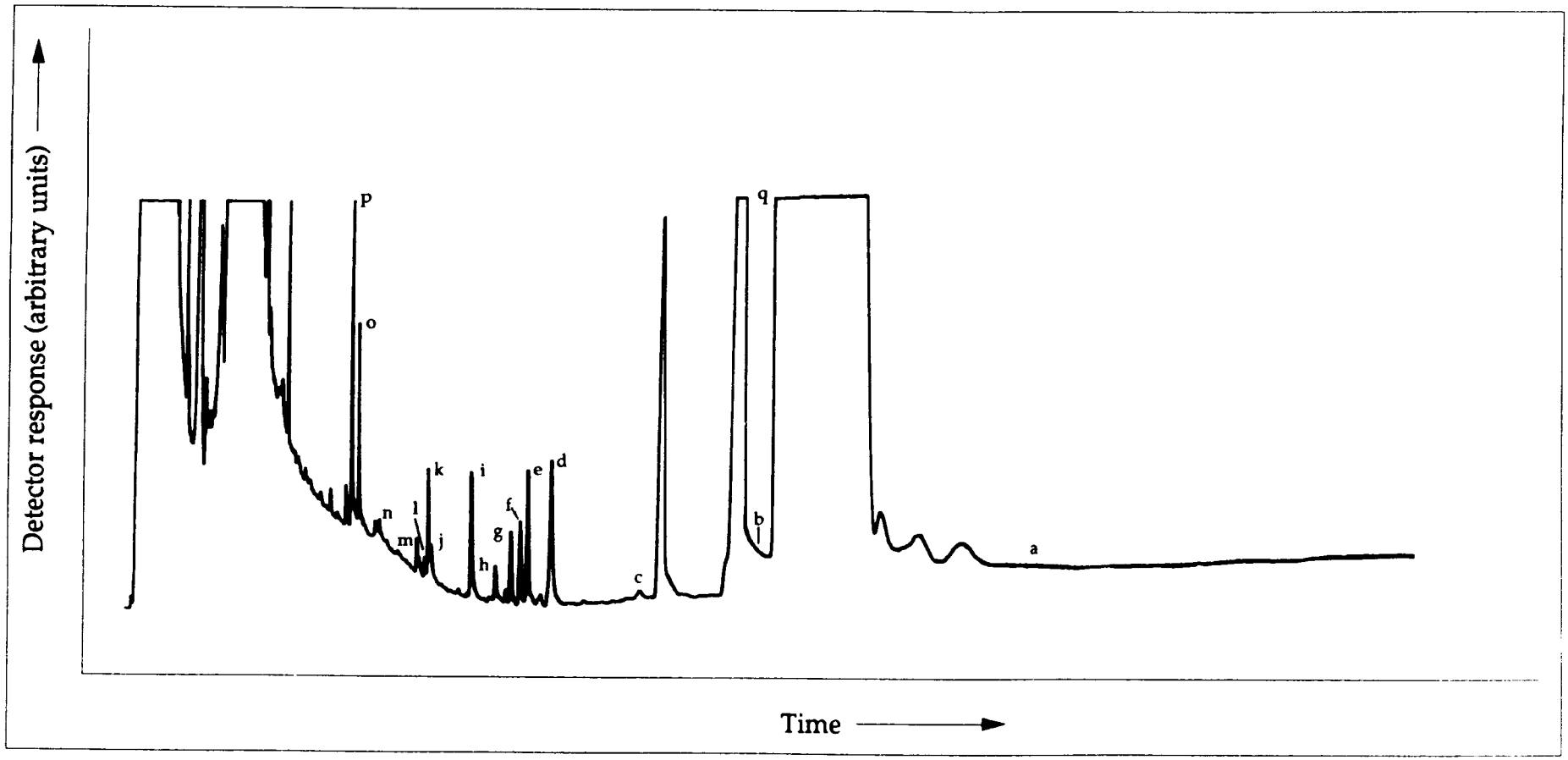


Fig. 4.2. Gas chromatogram of the components extracted from primary phase *C. perniciosa* and treated in accordance with the amino sugar analysis procedure. For full details see 4.1.1. Peak labels are those referred to in the main text.

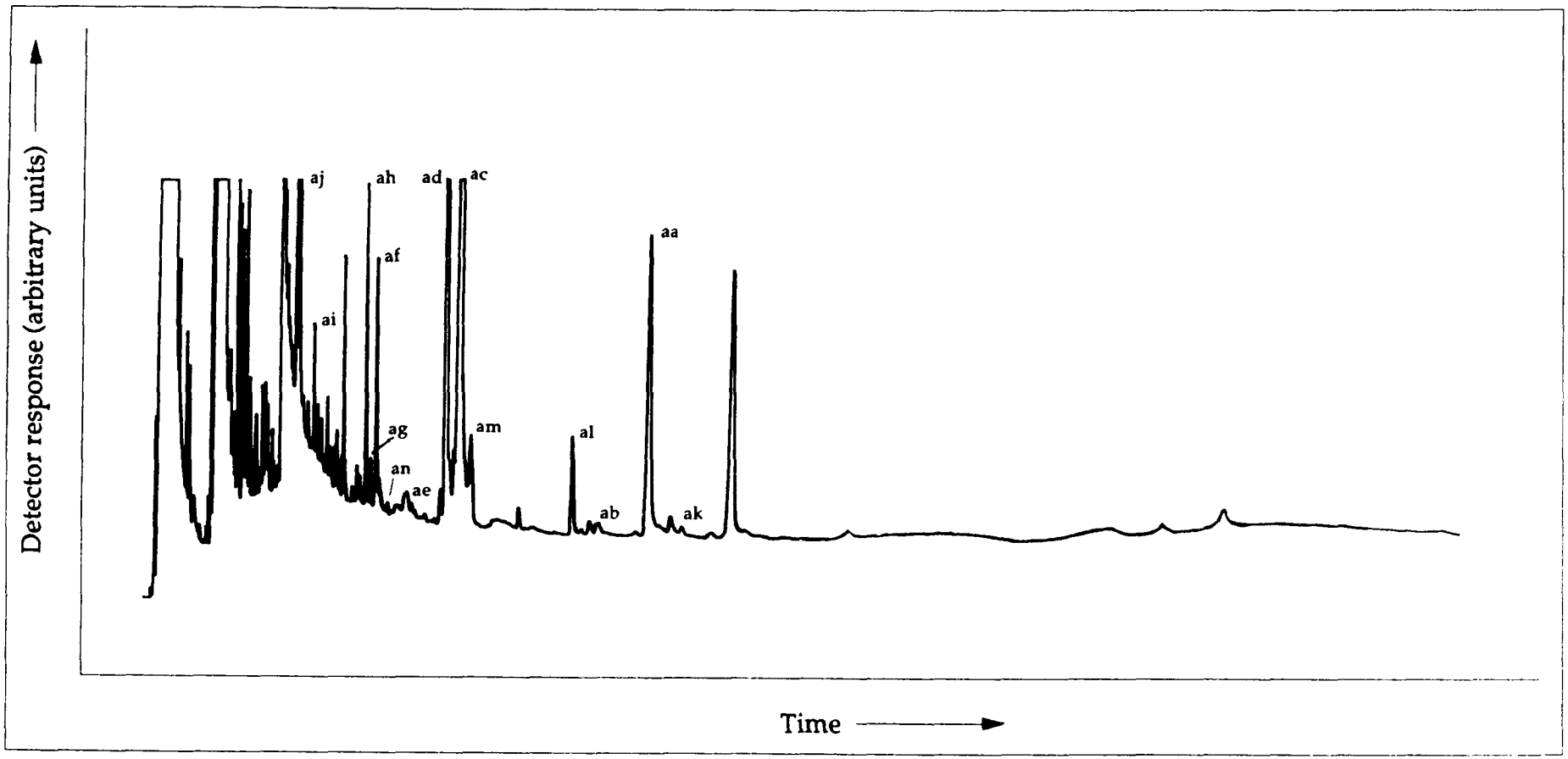


Fig. 4.3. Gas chromatogram of the components extracted from cocoa apical region tissue and treated in accordance with the neutral sugar analysis procedure. For full details see 4.1.1.

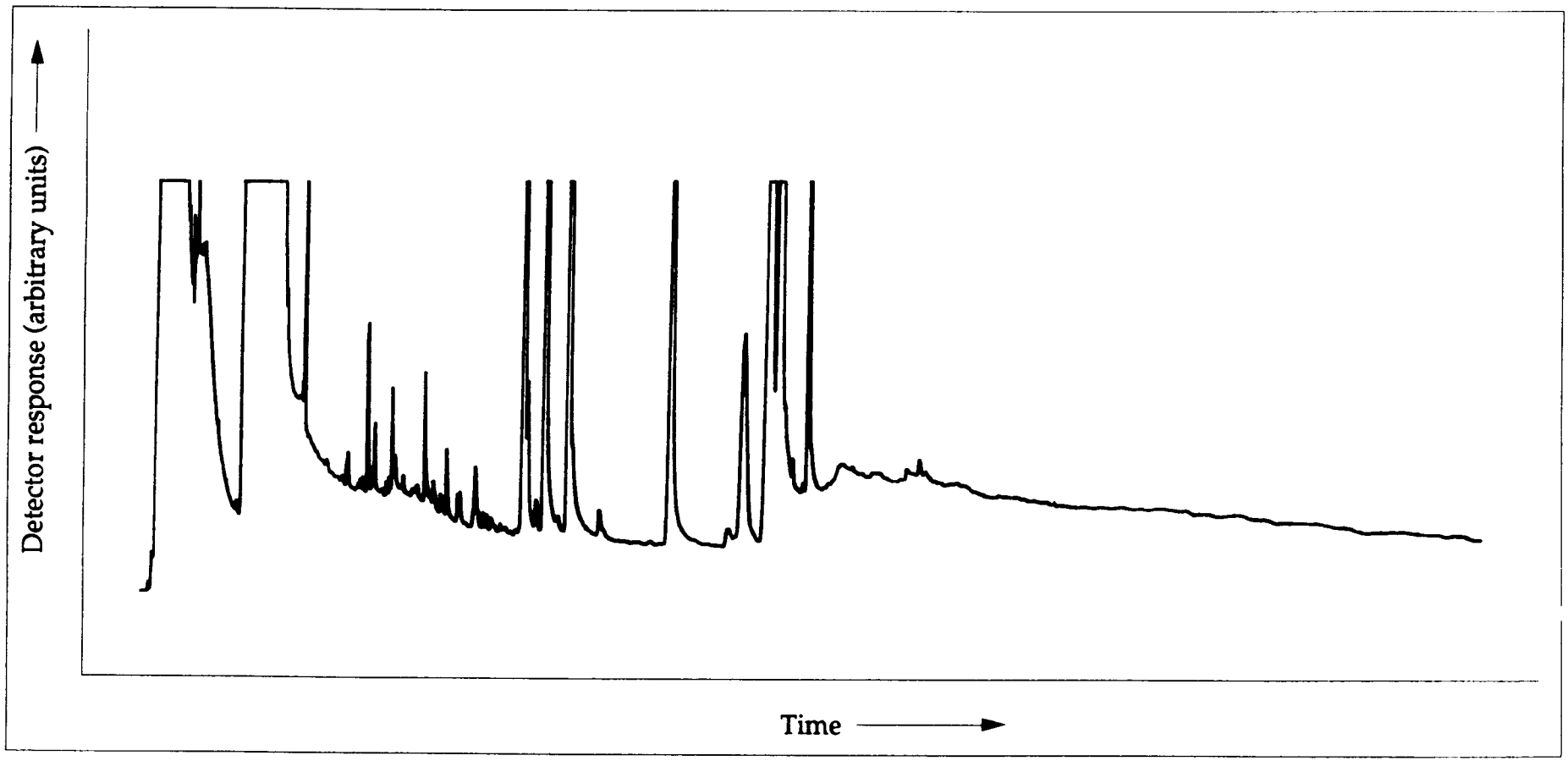


Fig. 4.4. Gas chromatogram of the components extracted from cocoa apical region tissue and treated in accordance with the amino sugar analysis procedure. For full details see 4.1.1.

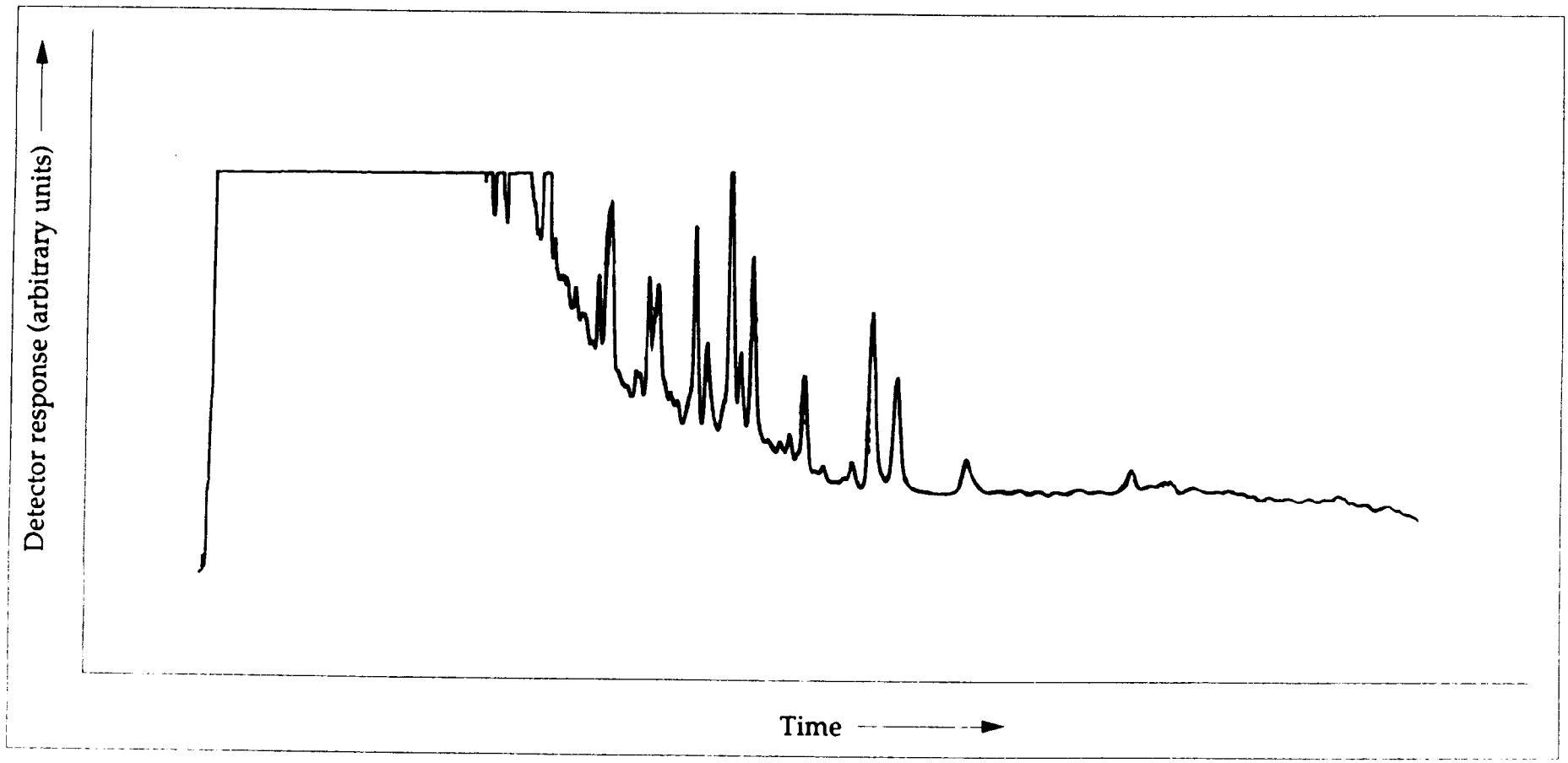


Fig. 4.5. The relative concentrations of components extracted from primary and secondary phase *C. perniciosa* and treated in accordance with the neutral sugar analysis procedure. For full details see 4.1.1. Standard deviations are indicated at the top of each bar.

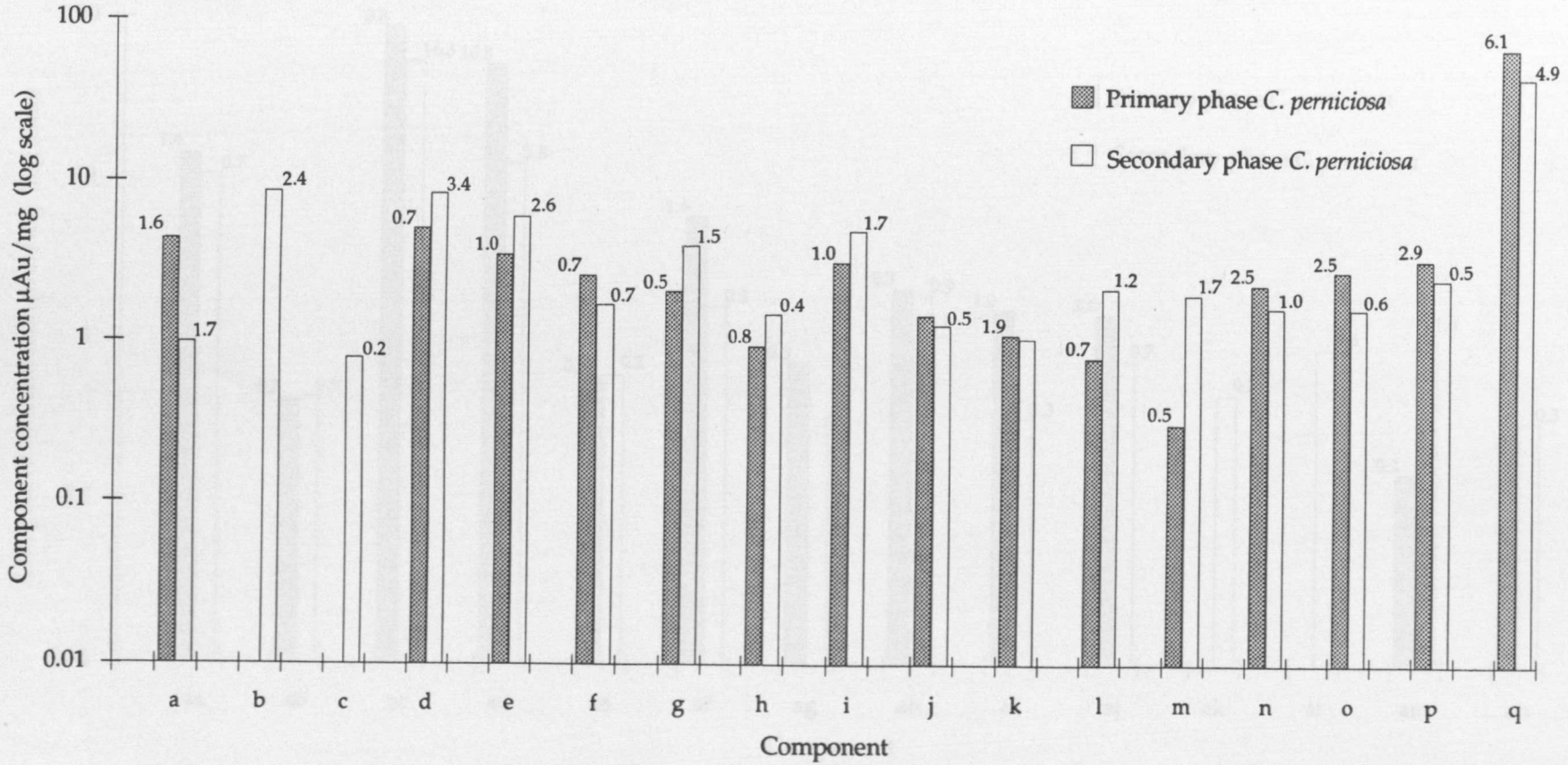


Fig. 4.6. The relative concentrations of components extracted from primary and secondary phase *C. perniciosa* and treated in accordance with the amino sugar analysis procedure. For full details see 4.1.1. Standard deviations are indicated at the top of each bar.

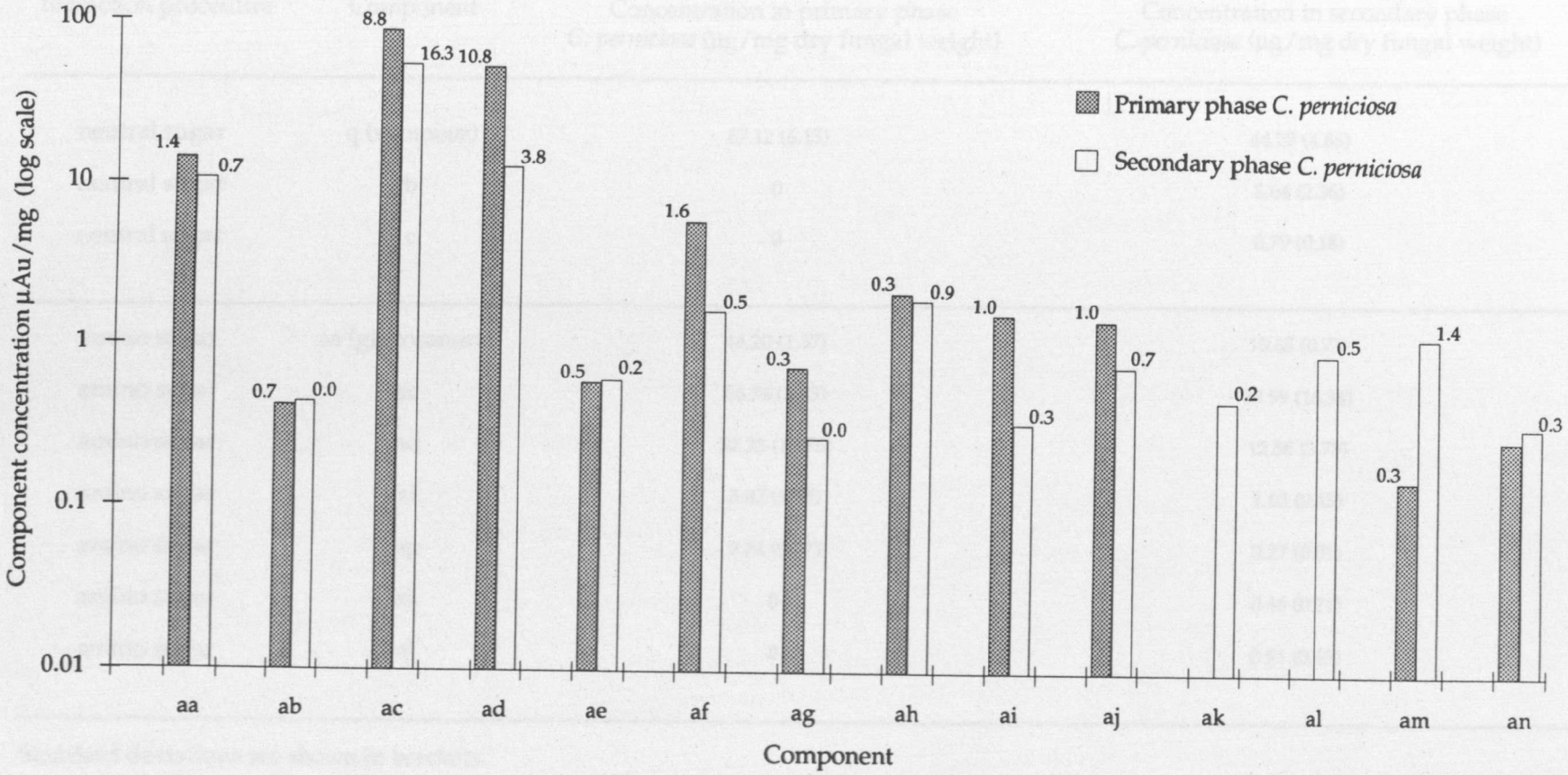


Table 4.1. Components exhibiting significantly different concentrations between the primary and secondary phases of *C. perniciosa*.

Extraction procedure	Component	Concentration in primary phase *C. perniciosa* (µg/mg dry fungal weight)	Concentration in secondary phase *C. perniciosa* (µg/mg dry fungal weight)
neutral sugar	q (mannose)	67.12 (6.15)	44.29 (4.85)
neutral sugar	b	0	8.64 (2.36)
neutral sugar	c	0	0.79 (0.18)
amino sugar	aa (glucosamine)	14.20 (1.37)	10.65 (0.73)
amino sugar	ac	86.74 (8.83)	53.99 (16.34)
amino sugar	ad	52.33 (10.75)	12.58 (3.78)
amino sugar	af	5.87 (1.57)	1.63 (0.45)
amino sugar	ag	0.74 (0.27)	0.27 (0.01)
amino sugar	ak	0	0.46 (0.21)
amino sugar	al	0	0.91 (0.49)

Standard deviations are shown in brackets.

Table 4.2. Component comparisons with significantly different ratios between the primary and secondary phases of *C. perniciosa*.

Component comparison	Ratio in primary phase *C. perniciosa*	Ratio in secondary phase *C. perniciosa*	F Ratio
q (mannose) * ad	1.3 (0.16)	3.65 (0.66)	35.7
mannose * af	11.81 (2.08)	27.97 (4.68)	29.91
mannose * ag	99.31 (33.71)	165 (13.92)	9.8
aa * ad	0.28 (0.09)	0.89 (0.23)	18.1
aa * af	2.57 (0.9)	6.83 (1.67)	15.04
aa * ag	22.21 (12.16)	39.74 (1.24)	6.172
ac * ad	1.69 (0.25)	4.3 (0.31)	125.6
ac * af	15.25 (2.76)	32.98 (1.41)	98
ad * af	9.02 (0.66)	7.68 (0.32)	9.85
ad * ag	75 (16.66)	46.88 (13.5)	5.16

Standard deviations are shown in brackets.

Table 4.3. Identification of components extracted from cocoa and primary and secondary phase *C. perniciosa* and treated in accordance with the amino and neutral sugar analysis procedures (see section 4.1.1). Components were identified using a mass spectrometer, as described in the main text.

Component code	Component name
b	galactose
d	a pentose
e	arabinose
g	β–D-ribose
q	mannose
aa	glucosamine
ac	glucose & galactose
ad	mannose
ah	β–D-ribose
ai	4, hexen-1-ol

Definite identifications for components 'd', 'e', and 'g' were possible only in cocoa tissue. Definite identifications for components 'ad', 'ah', and 'ai' were possible only in *C. perniciosa*. All other component identifications were possible in both cocoa and *C. perniciosa*.

Chapter 5

The lipid components of primary and secondary phase *C. perniciosa*

5.1.1a Introduction

The detection and quantification of fungal biomass using a fungal specific lipid is relatively well documented. The most commonly used lipids are the sterols, especially ergosterol. Seitz *et al.* (1979) explored the potential of ergosterol as a measure of fungal invasion in stored cereal grains. They found the assay a more sensitive and reliable indicator of early fungal invasion into cereal grains than a colorimetric chitin assay.

But not all fungi synthesise ergosterol, and organisms other than the fungi are capable of producing it. Several species of the green alga *Chlorella,* as well as some protozoa produce ergosterol, whereas the two fungal genera, *Pythium* and *Phytophthora* do not produce any sterols (Mercer, 1984). Lumsden *et al.* (1989) investigated the use of a variety of techniques including ergosterol assays as part of an investigation into the biocontrol of *Trichoderma harzianum* Rifai. The authors found that ergosterol content varied with the age of the fungal culture, and there was a significant increase in the ergosterol concentration at sporulation.

Martin *et al.* (1990) used an ergosterol assay as part of an investigation into the formation of ectomycorrhizas between *Eucalyptus globulus* ssp. *bicostata* Maid and *Pisolithus tinctorius* (Pers.) Coker & Couch. The authors found the assay extremely sensitive and it proved to be a useful investigative tool for monitoring early invasion of Eucalyptus roots by *P. tinctorius.*

The aims of this project required more than a biomass estimation procedure. Ideally, a method capable of estimating the relative proportions of the primary and secondary growth phases of *C. perniciosa* in infected cocoa tissue could be adapted or produced for use in the study of witches' broom disease. It was hoped this could be achieved by identifying lipid components specific to either growth phase, or by identifying significant differences in ratios between lipid components in the two growth phases.

Many workers have looked at differences in lipid composition between fungal species and strains. The lipid constituents of the Agaricales have been exhaustively investigated and reviewed by Solberg (1989). Senatore (1988) studied the sterol and fatty acid content of six

Agaricaceae and found few differences in the content of major lipid classes. However, the low concentration fatty acid components were found to differ significantly between species, and ß sitosterol was found only in relatively few species studied. Johnston & Coddington (1984) investigated the lipid content of several strains of *Schizosaccharomyces pombe*. The authors found substantial differences in lipid profiles between the wild type-strain and strains resistant to a variety of drugs. Two strains exhibited decreased levels of oleic acid and ergosterol, and an increased level of 24, 28, dehydroergosterol with respect to the wild-type. Thus potentially, there appear to be differences in lipid content between strains, and also differences in lipid or sterol ratios.

Clejan *et al.* (1986) also found species specific differences in lipid content. They found that *Bacillus subtilis* BD99 had a lower neutral to polar lipid ratio than two alkalophilic *Bacillus* strains. In addition, the *Bacillus* species had a greater variety of neutral lipids as well as squalenes and isoprenoids. Thus there appeared to be great variety in lipid content between a large number of fungal species, and this was true even for very closely related species.

Changes in lipid compositions in relation to infection have also been investigated. Rama Gopal & Sreenivasalu (1983) studied changes in total lipid content and individual lipids in relation to various infection stages in groundnut infected with *Arachis hypogaea* L. During disease development, total lipid, total lipid sugar, the ratio of total lipid sugar to lipid phosphorous, individual glycolipids and phosphotidyl glycerol, all decreased with respect to uninfected plants. The authors were attempting to relate easily measurable biochemical changes to different stages of disease development, hoping that a biochemical assay would substitute for a subjective visual test.

Age related changes in sterol phospholipid ratios during fruit ripening have also been noted. Lurie & Ben-Arie (1983) noted a major change in the sterol to phospholipid ratio in the climacteric and postclimacteric phases of ripening fruit. They also found that the ratio of saturated to unsaturated fatty acids increased during apple ripening. Itzhaki *et al.* (1990) found similar changes in the sterol to phospholipid ratio in senescing rose petal membranes.

Lipid ratio analysis has also been used for studying ageing in mammalian brains. For example, Saito *et al.* (1992) examined the ratio of cholesterol to phospholipids in nerve growth cones of the rat. The idea behind the experiments was to elucidate the biochemical characteristics of developmental changes. The authors found that the cholesterol to phospholipid ratio was well conserved during growth.

Latgé & Bièvre (1980), investigated the lipid compositions of *Entomorpha obscura* Hall & Dunn. Major changes were found in the neutral lipid composition with respect to growth phase, most notable was an increase in the lipid $C_{18:0}$. In contrast, the polar lipids appeared to vary much less throughout the various life cycle phases. The authors also found significant strain specific differences in lipid compositions.

Thus, there was a substantial body of opinion which indicated the presence of a wide variety of lipids in fungal tissues. And the work of Latgé & Bievrè (1980) indicated the possibility of differences in lipid composition between *C. perniciosa* growth phases. The earlier work, of Chapters 3 and 4, indicated that potentially useful ratios existed between sugar components, but unfortunately for various reasons they could not be used in cocoa. It was decided to expand this earlier work of chitin, mannan, neutral, and amino sugar analysis to include the lipids.

The following work was designed to determine the lipid profiles of the primary and secondary growth phases of *C. perniciosa* and cocoa apical region tissues. This, it was hoped, would allow the identification of lipids unique to *C. perniciosa*. It would also identify fungal growth phase specific lipids and any potentially useful lipid ratios. The principles behind ratio analysis have been discussed at length in Chapters 3 and 4. No work had previously been performed on the lipid composition of *C. perniciosa* and therefore any information gathered would prove to be interesting.

Procedures for lipid profiling

There are numerous methods for the estimation of the ergosterol content of a sample. Rao *et al.* (1989) developed a simple procedure that used a methanol extraction step, a TLC separation procedure, and an iodine based detection method. The procedure exploited the specific reaction of ergosterol and iodine. The product was then viewed under UV light at 271 nm. Unfortunately, the method required 50 g of starting material and had a detection limit of 500 ng. A method which used less than 25 mg of starting material was required to allow the sampling of defined cocoa regions and therefore this method was not used.

Salmanowicz & Nylund (1988) developed an ergosterol estimation procedure based on HPLC. The procedure required more than 300 mg of material and was therefore unsuitable for this project. Martin *et al.* (1990) improved the Salmanowicz & Nylund (1988) procedure to allow for sample sizes of less than 25 mg. However, a procedure based on HPLC was unlikely to provide the required high resolution between components. Very high resolution was required so that all possible lipid components could be quantified and identified. Another potential problem with the Martin *et al.* (1990) method was the detection procedure used. The procedure used UV detection at 280 nm and was therefore optimised for ergosterol estimation. Thus, it was unlikely that all the components separated chromatographically would be detected. Therefore it was decided to use a procedure based on gas chromatography which also gave the opportunity to use a mass spectrometer detector.

Methods which used GCMS were employed by Beilby & Kidby (1979) and by Beilby (1980), both used acetylated derivatives. The most sensitive and accurate method of assessing the sterol and lipid content of a sample of tissue is currently using GCMS (Goad pers. comm., Dept. Biochemistry, University of Liverpool, UK). Since Beilby & Kidby (1979) conducted their work there have been numerous advances in GC and MS design, as well as advances in derivatisation techniques. It was therefore decided to harness all the latest advances in GC, GCMS and derivatisation techniques for the aims of the project. These techniques, currently used by Goad and co-workers in the Department of Biochemistry, allowed the quantification of all major lipid components in *C. perniciosa* and cocoa.

Potential limitations of lipid analysis

There are two potential limitations to any lipid analysis procedure.

a) The lipid content estimated *in vitro* may not accurately reflect the concentration when the fungus is grown in the natural environment. Thus, a biomass estimation procedure based around the lipid may give a false result. However, if there are no other unforeseen factors then relative distribution of the fungus can be estimated.

b) As lipids are essentially very labile components in a fluid environment then concentrations can flux rapidly. Lipids are not as inherently stable as many other cellular components. For example, the lipid content, at least potentially, can flux very much quicker than the chitin content. This problem may become acute even over relatively short hyphal distances. This is an inherent flaw in any lipid analysis procedure and cannot be overcome.

5.1.1b Methods

Prior to extraction, fungal and plant materials were freeze-dried and stored in the dark under nitrogen gas at -20 °C. To each 20 mg sample, 20 µg of 5α–cholestanol internal standard was added. Lipids were extracted using 10 ml of redistilled chloroform/methanol (2:1 v/v). To facilitate lipid extraction, the samples were homogenised in a Potter's homogeniser for 1 min, with cooling in an ice bath every 30 seconds. Each extract was vacuum filtered through a glass fibre filter paper resting on a scintered glass funnel. The filter paper containing the sample residue was then re-extracted using a Potter's homogeniser and 10 ml of the chloroform/methanol mixture (2:1 v/v). The filtering and extraction steps were repeated, the sample filtered a final time, and the filtrate combined with the previous two filtrates.

Each total lipid extract was reduced in volume to 1 ml, then transferred together with washings to a 2 ml vial. The total lipid extract was reduced to dryness and stored in the dark under nitrogen at -20 °C. Extracts were stored under these conditions until immediately prior to derivatisation. Twenty microlitres of N, O, -bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TCSMA), and 20 µl redistilled pyridine were added to

the lipid extracts. The mixtures were then heated on a hot plate at 70 °C for 20 min. The derivatised mixtures were then dissolved in 50 µl of hexane and run on the GC. The samples were then diluted a further time with 500 µl of hexane and re-run on the GC.

Gas chromatography and mass spectrometry

Separation and quantification of lipid components was achieved on a Hewlett Packard 5890 Series 2 Gas Chromatograph fitted with a BP 5 capillary column. The BP 5 column was 25 M long, had a film thickness of 0.25 µm consisting of 5 % diphenyl dimethyl siloxane, and the column internal diameter was 0.22 mm. Helium was used as the carrier gas (1 ml/min), argon as the mixer gas (30 ml/min), the hydrogen had a flow rate of 30 ml/min and the air 430 ml/min. Peak areas were integrated using a Spectra Physics SP 4270 Integrator. Identification of components was achieved using a VG 7070H mass spectrometer equipped with a Finnigan INCOS data system. Two temperature programs were used to separate the different components. In the first GC run column conditions were as follows; 50-250 °C at 6 °C per min with an isothermal period of 45 min at 250 °C. In the second GC run, column conditions were as follows; 50-150 °C at 30 °C per min, then 150-285 °C at 6 °C per min with an isothermal period of 35 min at 285 °C. Each sample was run twice on the GC. The first time, the samples were diluted with 50 µl of hexane and analysed using the first temperature program; this allowed the separation and quantification of low concentration lipids. The second time, the samples were diluted with 500 µl of hexane and analysed using the second temperature program; this allowed the separation and quantification of the high concentration lipids including the sterols.

For sterol analysis, 5 α–cholestanol was an appropriate internal standard. Care was taken when interpreting the non-sterol lipid data. In many cases the internal standard differed chemically from the non-sterol components. Generally, to obtain a reliable estimate of the concentration of a specific component the internal standard should be chemically very similar to the component under study. Thus, 5 α-cholestanol could not be used to obtain definitive estimates of the concentrations of non-sterol components. But, it could be used to estimate the relative concentrations of the lipid components across different samples. The aim of this

study was to screen *C. perniciosa* tissues to elucidate the relative concentrations of lipid components in the primary and secondary phases of growth. The aim was to highlight the differences, not provide absolute concentrations of non-sterol components. When relative and not absolute non-sterol component concentrations were required, the method was adequate and the results accurate. The concentrations of the non-sterol lipid components were therefore given in arbitrary units. One mg was roughly equivalent to 1 Au and 1 ng was roughly equivalent to 1 nAu.

Samples analysed

Eighteen day old primary and secondary phase cultures of *Crinipellis perniciosa* grown as described in 2.1.1b were analysed for lipid components. Cocoa apical regions (the meristem, up to and including, 4 cm below the apical bud tip) from the cultivar Amelonado were also screened for lipid components.

Suppliers

Chloroform and methanol supplied by BDH Chemicals Ltd. BSTFA supplied by Pierce & Warriner. BP 5 column supplied by SGE Ltd. Hexane supplied by Fisons Scientific Ltd. The 5 α–cholestanol was kindly donated by John Goad, Department of Biochemistry, University of Liverpool, UK.

Statistical treatment of results

The central aim of the following analysis procedure was to highlight any components that could potentially form part of a useful ratio. This was done firstly, by identifying components that were found in significantly different concentrations in the two phases of *C. perniciosa*. And secondly, by identifying any components that exhibited little variation between life cycle phases and could therefore serve as a level from which other components could vary. The procedure was also designed to detect phase specific components, or any that may have served as biomarkers for *C. perniciosa*.

Step 1

Components with a concentration of more than 250 nAu/mg of dry fungal weight were highlighted on representative chromatograms obtained from samples of primary and secondary phase *C. perniciosa*. These peaks were then located and the components quantified on two other samples of primary and secondary phase *C. perniciosa*. The component concentrations were then assessed for significant differences in concentration between the primary and secondary forms of the fungus using a one way analysis of variance (ANOVA). The Null hypothesis was: the concentration of the component is identical in both growth phases of *C. perniciosa*. Thus, components which exhibited relatively large or random variations were excluded from the ratio analysis procedure. The components rejected by the ANOVA were treated in accordance with Step 3.

Step 2

The components not rejected by the ANOVA were then assessed to determine whether their concentrations varied significantly around a grand mean encompassing both growth phases. If the variation was small then they may have served as a base from which other components could vary, and thus potentially may have formed part of a useful ratio. The percentage variation was calculated for each component representing more than 250 nAu/mg of dry fungal weight on each reference chromatogram described above. A variation of 33 % or less from the overall mean encompassing both growth phases was considered acceptable, and certain not to miss any potentially useful components. These components were treated in accordance with Step 3. Components which occurred exclusively in only one growth phase were then analysed in accordance with Step 5. Thus, components with large or random variations in concentration were eliminated from the statistical analysis procedure.

Step 3

Ratios were then calculated for all possible combinations of the components highlighted in Steps 1 and 2. The resulting ratios were then assessed for significance using a one way ANOVA. The null hypothesis was: the ratio between the two components is identical in both

the primary and secondary growth phases of *C. perniciosa*. Ratios rejected by the ANOVA were considered to be potentially useful.

Step 4

The cocoa chromatograms for the extracts were then screened for the presence of either of the components which formed the potentially useful ratios.

Step 5

Components which occurred at more than 250 nAu/mg of dry fungal weight and found in only the primary or secondary phase were assessed for phase specific biomarker potential. Thus, potentially they could be used to estimate the concentration of either the primary or secondary phase in a mixture of the two. A variation of 33 % or less for all samples from the phase mean was considered acceptable. Chromatograms obtained from the extracts of cocoa tissue were then screened for the presence of the potential phase specific biomarkers.

Step 6

Components which occurred at more than 250 nAu/mg of dry fungal weight were assessed for fungal biomarker potential, i.e. could be used to estimate the *C. perniciosa* biomass in a sample of cocoa tissue. A variation of 33 % or less from the overall mean encompassing both growth phases was considered acceptable. This level of variation was considered generous and was certain not to miss any possibilities. Chromatograms obtained from the extracts of cocoa tissue were then screened for the presence of the potential biomarkers.

Step 7

The same reference samples described in Step 1 were then run on the mass spectrometer, as described earlier. Mass spectra were obtained for all possible peaks. The mass spectra obtained from the peaks in the samples analysed were compared to those in the inbuilt mass spectrometer library which contained more than 30,000 mass spectra. For each potential match the computer calculated a score out of 1000. Eight hundred or above was considered

sufficient to identify a compound. Therefore all peaks registering a score of 800 or more were considered to have been identified.

5.1.1c Results and Discussion

The chromatogram shown in Fig. 5.1 was obtained from primary phase *C. perniciosa* using the total lipid analysis procedure. The 32 peaks that represented more than 250 nAu/mg of dry fungal weight were highlighted with the letter codes 'a' - 'z' and 'aa' to 'ah'. Peaks 'f' and 'g' represented the internal standard on the two temperature programs. The results demonstrated the excellent component resolution obtained with the procedure despite the complexity of the derivatised extract. The chromatogram shown in Fig. 5.2 was obtained from secondary phase *C. perniciosa* using the total lipid analysis procedure. All the peaks that represented more than 250 nAu/mg of dry fungal weight were highlighted with the letter codes 'a'-'z' and 'aa' through to 'ah'. Peaks 'f' and 'g' represented the internal standard on the two temperature programs. Once again, the results demonstrated the excellent component resolution obtained using the total lipid analysis procedure. The chromatogram shown in Fig. 5.3 is a representative sterol profile of cocoa apical region tissue (Amelonado) obtained using the lipid analysis procedure. The chromatogram predominantly shows the sterols, which were very well separated and were therefore easily quantified. The sterols, where identified, were labelled accordingly.

The data presented in Fig. 5.4 and Fig. 5.5 illustrated the relative concentrations of the 32 different components extracted using the lipid analysis procedure and representing more than 250 nAu/mg of dry fungal weight in either primary or secondary phase *C. perniciosa*. Please note error bars could not be plotted accurately on the log scale graphs. Therefore, standard deviations were shown on top of each bar. Also, components 'f' and 'g' have not been plotted as they represent the internal standard for the two different temperature programs. The component identifications shown in Table 5.1 were obtained using the mass spectrometer's inbuilt data analysis and library system.

Steps 1 and 2

Using the total lipid analysis procedure, 32 peaks were identified as representing components with concentrations greater than 250 nAu/mg of dry fungal weight in either the primary or secondary phase tissue. The ANOVA identified two components with significantly different concentrations in either of the two growth phases. These components were 'h' and 'ae'. In addition, component 'd' failed the ANOVA but varied by less than 33 % around the overall component mean.

Step 3, 4 and 7

All possible ratios between the three components were calculated. Two ratios were found to be significantly different between the growth phases. These ratios were between 'd' and 'ae', and between 'h' and 'ae'. The ratio between 'd' and 'ae' in the primary phase was 14.97 (SD 6.92) and 1.28 (SD 1.12) in the secondary. The ratio between 'h' and 'ae' was 32.5 (SD 5.89) in the primary and 0.04 (SD 0.07) in the secondary phase.

Using the mass spectrometer, component 'h' was identified as squalene, component 'd' as ergosterol, while component 'ae' could not be identified.

The ratio between squalene and 'ae' would almost certainly have proved useful. The difference in the ratio between the two growth phases would probably have allowed the calculation of the relative concentrations of the two growth phases in infected cocoa tissue. Unfortunately, mass spectrometry demonstrated the presence of squalene and component 'ae' in the cocoa extracts. The detection of squalene was unsurprising as it is a precursor of many biological molecules, including the sterols. The ratio between ergosterol and 'ae' in the two different growth phases may also have proved useful. Ergosterol was not found in cocoa tissues but component 'ae' was. It was unsurprising to find ergosterol absent from the cocoa tissue but present in the fungal tissue because it is virtually a universal constituent of the fungi and produced by few other organisms, although certain plants are capable of producing it (Mercer, 1984).

Without a detailed mathematical approach the required difference between ratios which would have allowed the calculation of the relative proportions of primary and secondary phase *C. perniciosa* in an infected cocoa sample, could not be estimated. But the ratio between squalene and 'ae' was probably sufficiently different between the primary and secondary phases while that for ergosterol and 'ae' was probably not. Such mathematical rigour was not required in this instance because of itself it would have served no purpose. Thus, no usable lipid ratios were discovered, but the result validates the approach. In fact, the majority of components extracted and identified using the lipid analysis procedure were sugars. Sugars could not be definitively identified using mass spectrometry alone. Therefore, only the most probable identifications of the components were shown in Table 5.1.

Steps 5 and 6

No primary or secondary phase specific markers were found. The components were then assessed for fungal biomarker potential. Thirty one of the thirty two components were found to either occur in cocoa in significant concentrations relative to their concentration in *Crinipellis*, or varied by more than 33 % from their overall mean. The remaining component, ergosterol, passed all the criteria and may therefore be used as a biomarker.

Ergosterol was a major lipid component of both life cycle phases of *C. perniciosa*. The mean concentration of ergosterol in the tissues studied was 1.66 μg/mg of dry fungal weight (SD 0.33). Other workers have found similar ergosterol levels in other fungi. For example, Martin *et al.* (1990) estimated the ergosterol concentration as 1.14 μg/mg of dry fungal weight (SD 0.21) and 1.58 μg/mg of dry fungal weight (SD 0.25) for *Laccaria laccata* (Scop. :Fr) Berk. & Broome and *P. tinctorius* respectively. Seitz *et al.* (1979) estimated the ergosterol concentration as 2.3 μg/mg of dry fungal weight (no SD was quoted) in *Aspergillus flavus*.

However, the ergosterol concentration in *C. perniciosa* ranged from 1.3-2.1 μg/mg of dry fungal weight. The relative level of variation for ergosterol between phases was slightly greater than the values obtained using the newly developed, highly sensitive and specific chitin assay (12.42 μg/mg of dry fungal weight SD 2.18). In addition, intuition would suggest,

chitin would be a more stable biomarker as it is a structural component of the cell wall rather than being a labile lipid in the fluid cell membrane. Chitin is also less labile and is therefore easier to handle in an experimental situation. Ergosterol is sensitive to light, heat, and oxygen. Therefore, for quantitative work extreme care needs to be taken (Goad pers. comm.). In the field, dead or dying infected material will be in an environment containing high temperatures, light and oxygen levels. Therefore, there would be some doubt as to whether the ergosterol estimate obtained would accurately reflect true fungal loading. This criticism also applied to the chitin assay (Sharma *et al.*, 1977). However, a chitin assay can be used to estimate relative distribution of fungus within the host, but factors such as shading could dramatically alter the ergosterol concentrations over relatively small distances and time-scales. In addition, the experimental material obtained would need to be treated with the utmost care so as to not accentuate the problems associated with ergosterol lability. Chitin, which is inherently more stable would not suffer to the same degree from these problems. Thus, the chitin analysis procedure would probably be a more reliable biomass estimation protocol. However, at extremely low levels of infection ergosterol estimation using GCMS and selected ion monitoring may prove more effective.

5.1.1d Conclusions

A wide range of lipid components were quantified in primary and secondary phase *C. perniciosa*. Two potentially useful lipids were discovered. At least one of these ratios would have proved useful. But unfortunately, both ratios contained at least one component which occurred in cocoa tissue. Therefore, in this instance no usable lipid ratios were discovered, but the result validated the approach.

Ergosterol appeared to be a major lipid component of both growth phases of *C. perniciosa*. The mean concentration of ergosterol in *C. perniciosa* was about 1.66 µg/mg of dry fungal weight (SD 0.33). An ergosterol assay probably would not form the basis of a reliable quantitative estimation procedure for *C. perniciosa* in cocoa tissue. But, where cocoa or other plant materials need only be screened for the presence of fungal material then the procedure using GCMS would likely prove to be a particularly sensitive approach.

Fig. 5.1. Gas chromatogram of the components extracted from primary phase *C. perniciosa* and treated in accordance with the total lipid analysis procedure. For full details see 5.1.1. Peak labels are those referred to in the main text.

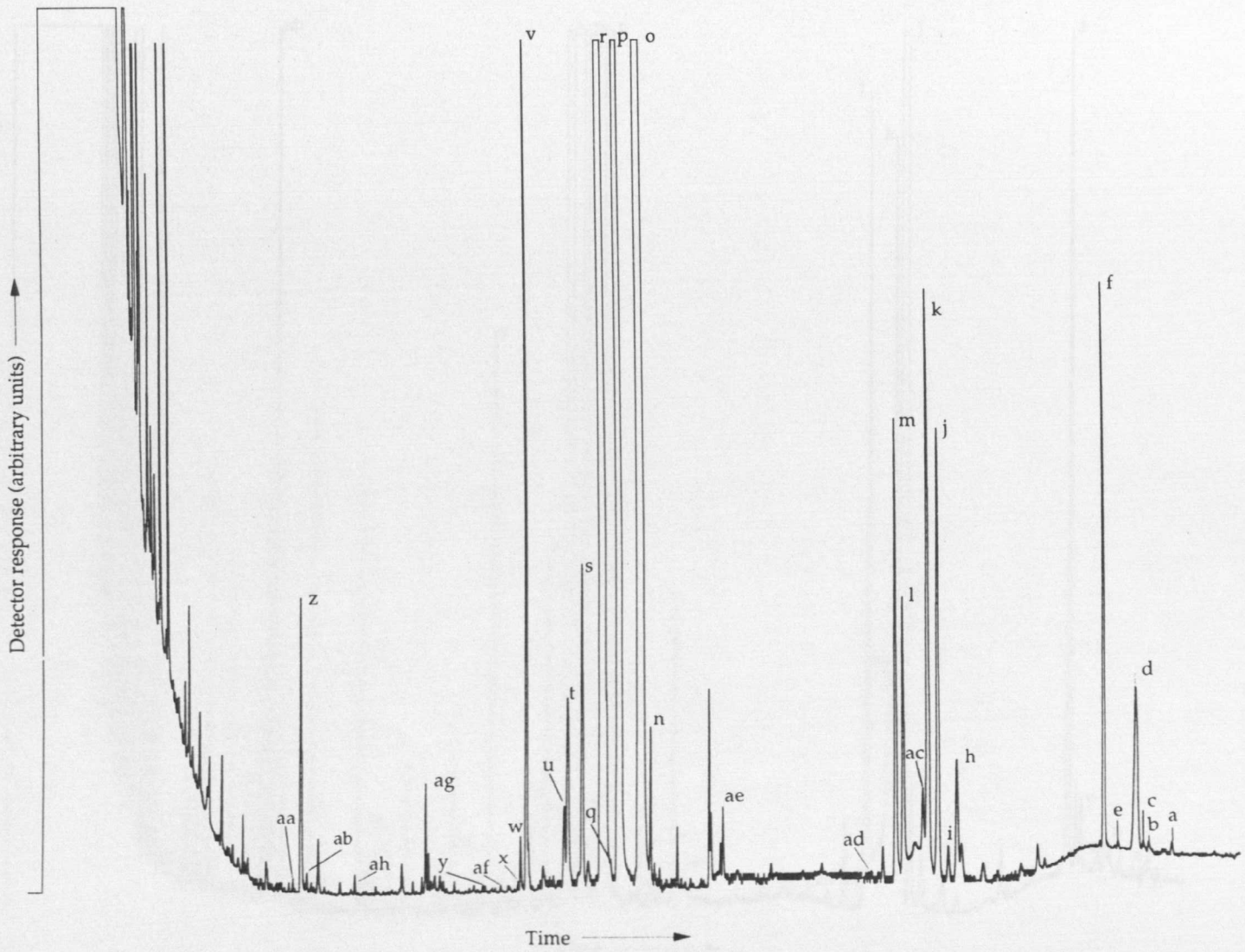


Fig. 5.2. Gas chromatogram of the components extracted from secondary phase *C. perniciosa* and treated in accordance with the total lipid analysis procedure. For full details see 5.1.1. Peak labels are those referred to in the main text.

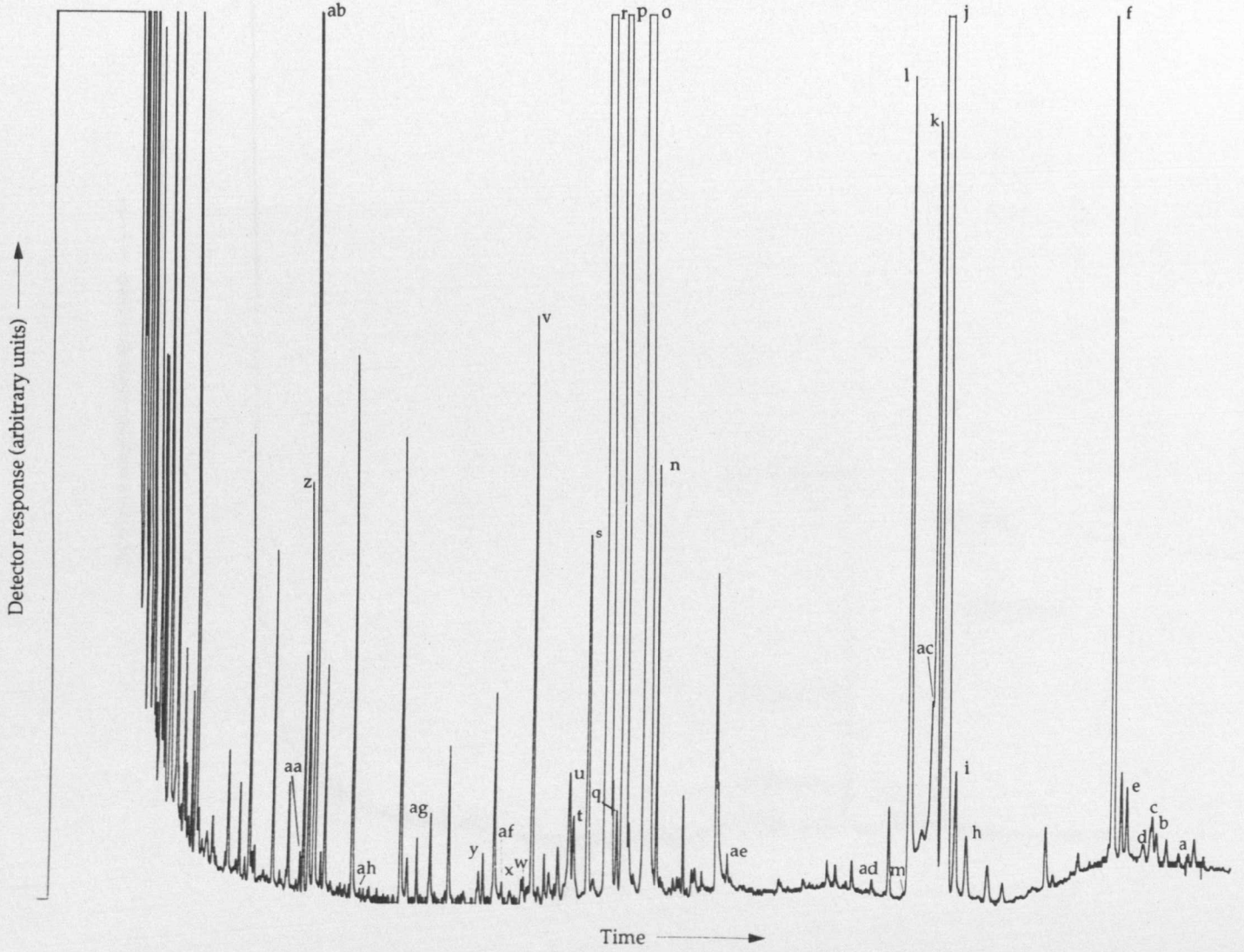


Fig. 5.3. Gas chromatogram of the sterols extracted from cocoa apical region tissue and treated in accordance with the total lipid analysis procedure. For full details see 5.1.1. The sterols, where identified, are labelled accordingly.

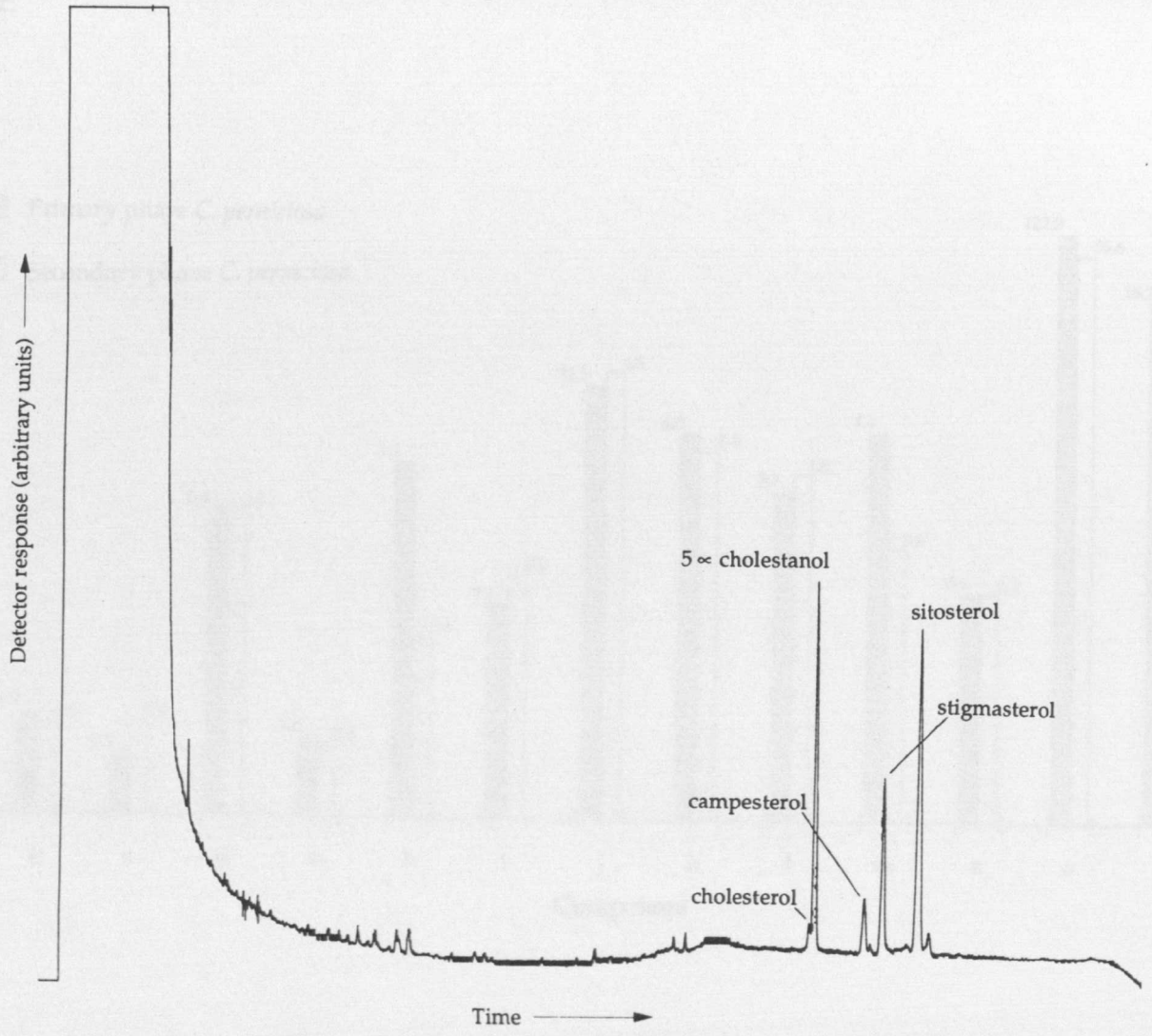


Fig. 5.4. The relative concentrations of components 'a' - 'r' extracted from primary and secondary phase *C. perniciosa* and treated in accordance with the total lipid analysis procedure. For full details see 5.1.1. Standard deviations are indicated at the top of each bar.

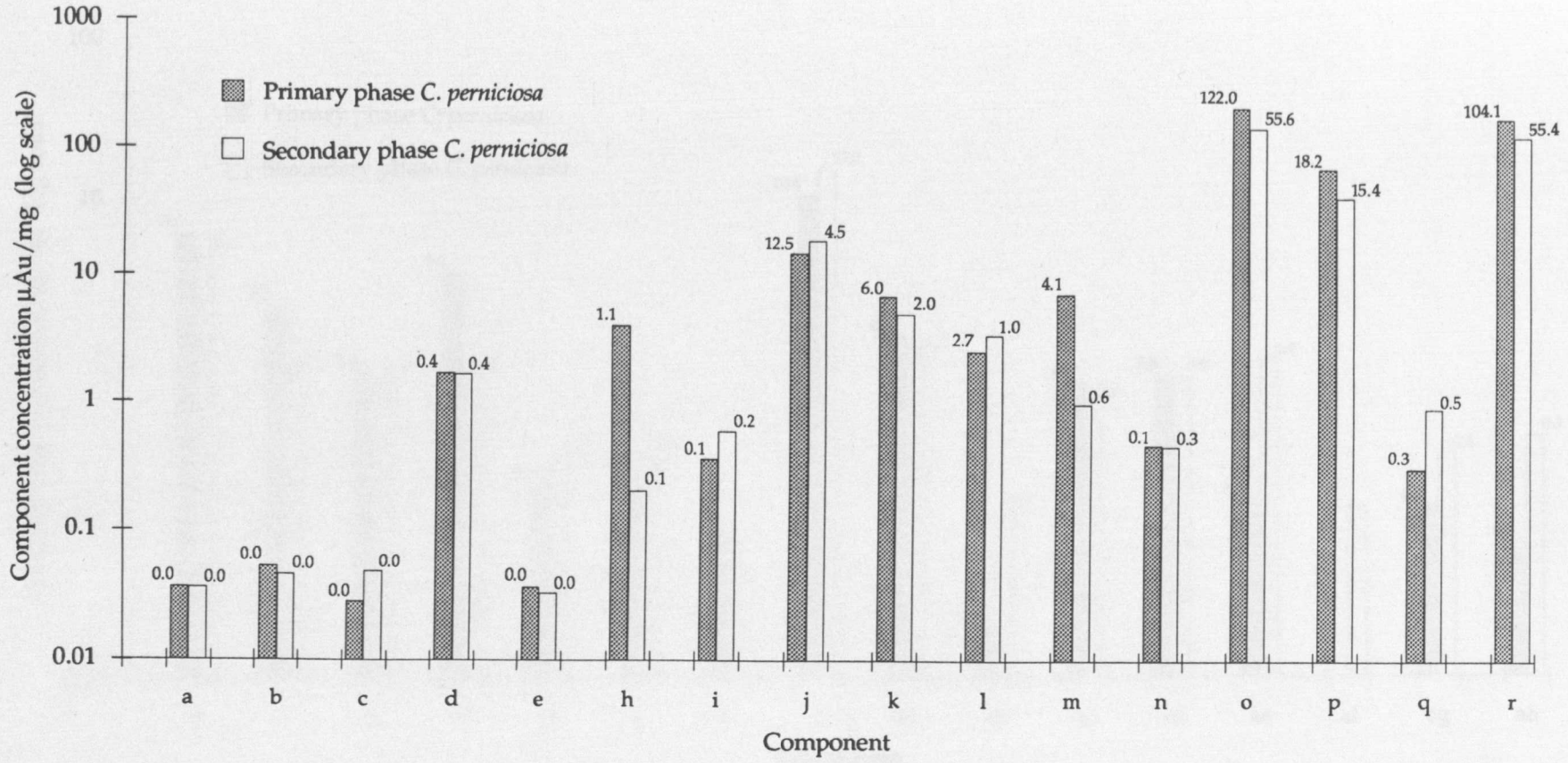


Fig. 5.5. The relative concentrations of components 's' - 'ah' extracted from primary and secondary phase *C. perniciosa* and treated in accordance with the total lipid analysis procedure. For full details see 5.1.1. Standard deviations are indicated at the top of each bar.

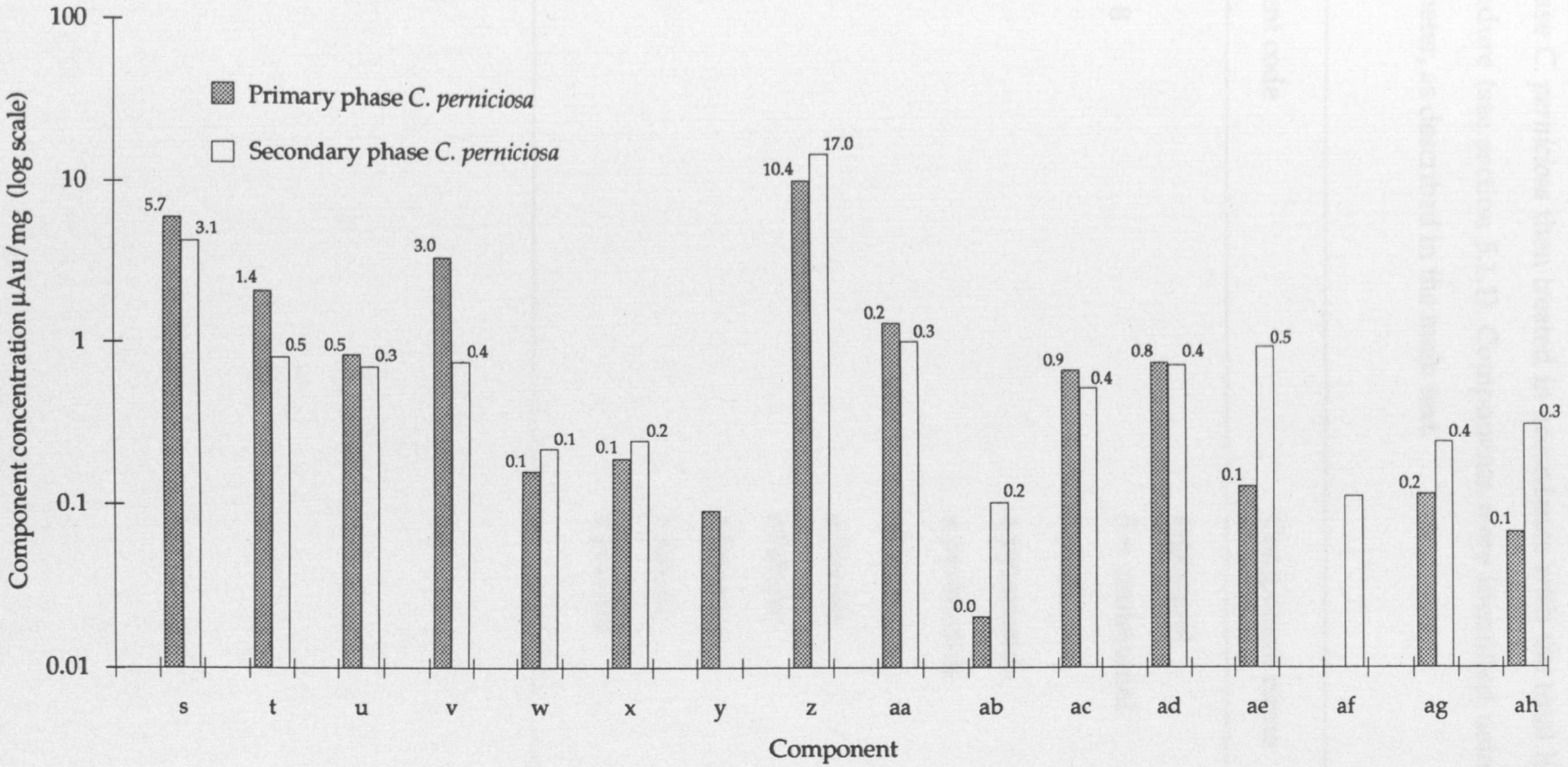


Table 5.1. Identification of components extracted from cocoa and primary and secondary phase *C. perniciosa* then treated in accordance with the total lipid analysis procedure (see section 5.1.1). Components were identified using a mass spectrometer, as described in the main text.

Component code	Component name
d	ergosterol
f & g	5 ∝ cholestanol
h	squalene
j	a pyranoside
k	a pyranoside
l	a hexose
m	a hexose
o	a hexose
p	a hexose
r	a hexose
s	a pentitol

Chapter 6

Investigations using the new chitin assay

6.1.1a Introduction

The work covered in Chapters 3 and 4 indicated that a new and highly sensitive chitin assay had been successfully developed. The work in Chapter 5 showed this assay to be a more sensitive and useful indicator of fungal biomass in infected cocoa than an ergosterol assay. One of the original aims of the project was to develop a biochemical method of estimating the relative proportions of the primary and secondary forms of *C. perniciosa* in infected cocoa tissues. Unfortunately, a phase specific biomarker was not discovered, nor was a ratio between different fungal components that might have allowed the apportioning of the relative concentrations of primary and secondary phase *C. perniciosa* in infected cocoa tissues. However, since the chitin assay appeared to offer great potential as a fungal specific biomarker, it was decided to use the new procedure to study cocoa tissues infected with *C. perniciosa.*

Previous workers have found the chitin concentration in fungi may change with physiological age (Sharma *et al.*, 1977). However, Whipps *et al.* (1985) countered the criticisms of Sharma *et al.* (1977) by using a chitin assay for comparative purposes only. Therefore, the aim of the initial work in this chapter was to investigate the effect of ageing on the fungal chitin content, and also to determine whether the chitin content varied between the primary and secondary phases of *C. perniciosa*. This was achieved by measuring the chitin concentration in cultures of primary and secondary phase *C. perniciosa* of differing ages. If the chitin content varied erratically and significantly then the potential usefulness of the chitin assay would be correspondingly reduced. A further aim of this initial work was to determine a conversion factor that would allow the quantity of fungal hyphae to be calculated from a measure of chitin content.

Little is known about the mechanisms of broom formation. For example, the Chigorodo isolate of *C. perniciosa* infects the cocoa cultivar EET 400 and causes the formation of grossly distorted stems and suppresses side shoot development, while other fungal isolates induce the formation of the more typical brooms (Wheeler, 1985). A representative broom is shown in 6.1. No mechanism has been found in cocoa to allow for the differences in response to

infection by *C. perniciosa*. Broom formation is believed to be linked to changes in concentrations of plant growth regulators, but Orchard *et al.* (1993) found no fluxes in levels during broom formation. However, the concentration of zeatin riboside, the presumed inactive bound form of zeatin, was found to differ significantly in broom tissues in relation to uninfected tissues.

There also appears to be some doubt as to whether significant levels of *C. perniciosa* biomass are necessary to induce disease symptoms (Wheeler, 1985; Hardwick pers. comm.) Obvious disease symptoms are frequently present where little or no fungal hyphae are visible in tissue sections viewed by microscopy (Collin pers. comm., Department of Genetics & Microbiology, University of Liverpool, UK). There is also little evidence to suggest brown brooms contain more fungal hyphae than green brooms, although Rocha (1983) estimated approximately four times as much *C. perniciosa* biomass in brown brooms than in green. It is also possible that broom formation may be induced by a translocatable factor and thus the direct presence of *C. perniciosa* may not be necessary to induce broom formation. Therefore, the work in this chapter was also designed to determine whether detectable concentrations of fungal hyphae were present in a variety of infected brooms, and whether the concentrations varied between green and brown brooms. This information was required to determine whether *C. perniciosa* induces broom formation by its localised presence in broom tissue, or whether the brooms are induced by a more general systemic effect. Answering these questions is vital if phytosanitation is to be optimised. Phytosanitation is the process whereby visibly infected material is removed from cocoa trees. If *C. perniciosa* induces a more general systemic reaction in the host then it is possible that far more extensive pruning may be required than if, as presumed, the *C. perniciosa* hyphae are relatively localised in the broom area. More extensive pruning would be required to ensure all *C. perniciosa* hyphae were removed from cocoa during phytosanitation.

In addition, Rocha (1983) using the colorimetry based assay, discovered that in both green and brown brooms, more *C. perniciosa* was present at the nodes than the internodes. It was decided to build on this work by using the newly developed chitin assay. The new assay had

proved capable of analysing sub-microgram portions of tissues and would therefore allow extremely specific areas of cocoa tissues to be analysed. The remaining work of this chapter was therefore designed to elucidate the distribution of *C. perniciosa* within a single broom. This was achieved by estimating the *C. perniciosa* distribution, section by section, throughout the length of an entire broom. Analytical work was augmented by a detailed scanning electron microscopy study (SEM) which provided clues to the distribution and growth patterns of the fungus. Only this approach would indicate whether the fungus was growing intra- or intercellularly at a particular point in the broom.

6.1.1b Methods

The variation in chitin concentration with age in primary and secondary phase *C. perniciosa*

Samples of 12, 18, and 25 day old cultures of primary phase *C. perniciosa* grown as described in 2.1.1b. were treated in accordance with the new chitin estimation procedure described in 3.3.2b. Samples of 11, 21, 27, 35, and 41 day old secondary phase *C. perniciosa* cultures grown as described in 2.1.1b were also treated in accordance with the new chitin estimation procedure described in 3.3.2b. Identification of chitin was achieved by co-chromatography and mass spectrometry.

The *C. perniciosa* content of green and brown brooms

Green and brown brooms, 5 of each, were obtained from the cultivar Amelonado grown at the Cocoa Research Unit, The University of the West Indies, St Augustine, Trinidad. The brooms all consisted of at least 4 main shoots and numerous side branches. The brooms were frozen on site, then transported and freeze-dried in Liverpool within 24 hours. The samples were ground in a mortar and pestle, then treated in accordance with the chitin analysis procedure, as described in section 3.3.2b.

The distribution of *C. perniciosa* throughout a green broom

Basidiospores from *C. perniciosa* Manaus F were collected as described in 2.1.1b except the potato dextrose liquid was replaced by a collecting fluid consisting of 16 % (v/v) glycerol and

0.01% (v/v) Tween 20 in 0.01 M MES buffer at pH 6.1. The Tween 20 was added after autoclaving. The spore suspension was concentrated using a 0.2 µm filter and the concentration adjusted to 1 million spores/ml. The spores were then either stored under liquid nitrogen or used immediately. Prior to use, the spores were diluted with Tween 20 (0.01 % v/v) containing MES buffer at pH 6.1, to ensure the glycerol concentration was less than 3 % v/v.

Brooms were produced using the method of Wheeler (pers. comm.). Aliquots (50 µl) of the spore suspension containing 5-10,000 spores were pipetted onto 1.5 % (w/v) water agar plugs (3 mm dia) and allowed to dry for 30 min. The inoculated water agar plugs were then placed over young cocoa buds (1-2 mm dia), covered in damp absorbent cotton wool and overlaid with cling film. After 24 hr, the cotton wool and cling film were removed, leaving as much of the agar plug in place as possible.

After 12 weeks one of the resulting brooms was harvested. The broom was green, with distorted growth and large necrotic patches, and may have been in the process of turning into a brown broom.

Two transverse sections (2 mm high) were taken from the base of the broom (indicated in Fig. 6.5). The first of the transverse sections was freeze-dried, ground in a mortar and pestle, and treated in accordance with the chitin analysis procedure outlined in 3.3.2b. The second transverse section was taken for the SEM study. This sectioning process was repeated every 1 cm from the base of the broom to the meristem. A highly detailed profile of the entire broom was therefore built-up. In addition, the vascular tissue from below the base of the broom, and a distorted bud 4 cm from the base were also removed for analysis.

The sections for the SEM study were dehydrated in pre-cooled absolute ethanol at -4 °C overnight. The alcohol was then replaced by more absolute ethanol at -4 °C and stored at 4 °C for 24 hours. The sections were then allowed to reach ambient temperature.

The sections were then critical point dried in a Polaron E3000 Critical Point Drier using CO_2 (supplied by Distillers UK Ltd.). The sections were then glued to stubs and sputter coated with 60 % gold/palladium (supplied by Bio-Rad UK Ltd.) in a Polaron E5100 Sputter Coating Unit. The specimens were then viewed in a Philips 50/B Scanning Electron Microscope using an accelerating voltage of 7.2-15 KV.

6.1.1c Results and Discussion

The variation in chitin concentration with age in primary and secondary phase *C. perniciosa*

The data presented in Fig. 6.1 clearly illustrated a steady decline in chitin concentration when primary phase *C. perniciosa* aged. The data was illustrated as a scattergram and therefore no standard deviations were shown. The mean chitin concentration at day 12 was 19.07 µg/mg of dry fungal weight (SD 3.99), this declined to 14.83 µg/mg of dry fungal weight (SD 0.77) by day 18 and by day 25 the mean concentration had declined to 9.30 µg/mg of dry fungal weight (SD 1.50). The overall mean concentration for all the sampling times was 14.40 µg/mg dry fungal weight (SD 4.90). The previous work (section 3.3.2c) showed that the primary phase chitin concentration was 14.83 µg/mg of dry fungal weight (SD 0.77). These two sets of results compared very well, indicating strongly that a conversion factor based upon this chitin value would be likely to reflect the true level of primary phase *C. perniciosa* in a sample.

The data presented in Fig. 6.2 clearly illustrated that during the growth of secondary phase *C. perniciosa* in liquid culture, the chitin concentration increased up to day 21 and then declined slowly to day 41. Once again, the data was illustrated as a scattergram and therefore no standard deviations were shown. At day 11, the mean chitin concentration was 12.18 µg/mg of dry fungal weight (SD 3.22) and this increased to 32.85 µg/mg of dry fungal weight (SD 2.23) by day 21. The concentration then declined to 24.31 µg/mg of dry fungal weight (SD 4.14) by day 35 and remained relatively stable at 17.43 µg/mg of dry fungal weight (SD 8.61) by day 41. These results were in contrast to those of primary phase *C. perniciosa*. In the primary phase there was a steady decline in the chitin concentration as ageing progressed, whereas the results obtained for the secondary phase indicated an increase in chitin

concentration up to day 21 and then a decline in chitin concentration as ageing progressed. The mean chitin concentration at day 27 was significantly different from the values for the other ages. The value of 10.61 μg/mg of dry fungal weight (SD 1.00) is out of step with the general rising, and then falling trend in chitin concentration.

The mean concentration of chitin in the secondary phase over all ages examined was 19.48 μg/mg of dry fungal weight (SD 9.20). These results were higher than the concentration of 14.40 μg/mg of dry fungal weight (SD 4.90) previously obtained for the primary phase. The results were comparable, if somewhat higher, than those previously obtained for secondary phase *C. perniciosa*. Our work shown in 3.3.2c indicated that the mean chitin concentration in the secondary phase was 17.02 μg/mg of dry fungal weight (SD 0.63). Thus, it was likely that a conversion factor based upon this value would likely reflect the true level of secondary phase *C. perniciosa* in a sample.

Using a biochemical based technique alone, it is not possible to distinguish between primary and secondary phase *C. perniciosa* in infected cocoa. Therefore, the chitin concentration must only serve as a guide to total fungal content in host tissue. The mean concentration for all primary and secondary phase *C. perniciosa* samples analysed in the two experiments was 16.36 μg/mg of dry fungal weight (SD 7.55). Thus, the conversion factor was 61.12 (SD 28.32). For each sample mean, the estimated concentration of chitin was therefore multiplied by 61.12 to obtain the *C. perniciosa* concentration. The standard deviation of the resulting estimate of *C. perniciosa* concentration was obtained using the formula:

$$\sqrt{(\text{conversion factor SD}^2/\text{conversion factor mean}^2) + (\text{sample SD}^2/\text{sample mean}^2)}$$

Rocha (1983) estimated the chitin content of *C. perniciosa* to be 8.52 μg/mg of dry fungal weight, no SD was reported. Therefore, Rocha's (1983) data produced a chitin to *C. perniciosa* conversion factor of 117.37. Therefore, Rocha's (1983) estimates of the chitin content of *C. perniciosa*, the conversion factor, and the background glucosamine concentration in cocoa were somewhat different to those obtained from the work in this thesis. The new procedure

developed in 3.3.2c did not suffer from the problems associated with the traditional colorimetry based procedure. Thus, the conversion factor calculated from the experiment outlined above is likely to furnish the most accurate assessment of fungal loading in infected cocoa tissue currently obtainable. In addition, Rocha's (1983) work was performed on *C. perniciosa* isolates from Chigorodo (Colombia), Pichilingue (Ecuador), Ouro Preto (Brazil), and Trinidad, whilst this work was performed on an isolate from Manaus (Brazil). At least part of the difference in the conversion factor may be due to differences in the chitin content of different *C. perniciosa* isolates.

Earlier work (3.3.2c) indicated that chitin, probably as glucosamine, was present in low concentrations in cocoa tissues. The concentration was in the order of 30 ng/mg of dry sample weight. Thus, a reliable quantification limit for *C. perniciosa* in cocoa tissue was about 0.3% w/w. Hence the *C. perniciosa* content could only be reliably estimated if it constituted more than about 0.3% w/w of cocoa tissue. This did not prove to be a problem.

The *C. perniciosa* content of green and brown brooms

The results (Table 6.1) clearly showed that chitin, and therefore fungus, was present in significant quantities in both green and brown brooms. A representative broom is illustrated in Fig. 6.3. It was reasonable to assume that most chitin originated from *C. perniciosa.* The fungus was present in all green and brown brooms tested, this indicated strongly that broom formation was linked to the localised presence of *C. perniciosa,* rather than resulting from a more general systemic effect. A more general systemic effect could be caused by an unknown factor produced by *C. perniciosa* and carried throughout the cocoa plant. This factor could then induce broom formation without the localised presence of *C. perniciosa* in the broom tissue. If little or no fungus had been detected in one or more of the 10 brooms analysed, this would have suggested broom formation was linked to a more general systemic effect. These results indicated localised *C. perniciosa* hyphae were associated with broom formation.

The average *C. perniciosa* content in the green brooms was 81.48 μg/mg of dry sample weight (SD 0.46). Whereas the average *C. perniciosa* content in the brown brooms was 160.97 μg/mg

of dry sample weight (SD 0.46). Thus, green brooms contained less *C. perniciosa* biomass than brown brooms. Rocha (1983) also found green brooms contained less fungal biomass than brown brooms. Unfortunately, no direct numerical comparison was possible between this work and Rocha's (1983), because of significant differences in sampling procedures, but on average, the *C. perniciosa* content of green and brown brooms was approximately 75 and 320 µg/mg of dry sample weight respectively. Rocha's (1983) results do not correlate well with those outlined here. However, for the reasons outlined earlier the new chitin assay is likely to produce more accurate results, but they did show the same trend of green brooms to contain less *C. perniciosa* biomass than brown brooms.

It was unlikely that fungal loading alone caused the death of green brooms and formation of brown. The difference in fungal content between green and brown brooms was insufficient to allow for such major changes in the plant. Another factor, other than fungal loading must have induced necrosis in green brooms and the consequent formation of brown brooms. Evans (1980) proposed that two factors were responsible for the major characteristics of witches' broom. The first factor was believed to be involved with fungal biotrophy and green broom production. This factor was presumed by Evans (1980) to be auxin inducing. The second factor was believed to be associated with saprotrophy and the formation of brown brooms, and was presumed to be an auxin destroying factor. However, Orchard *et al.* (1993) found no imbalances in plant growth regulators in a variety of witches' brooms. Thus, it is likely that *C. perniciosa* does not produce distorted growth or green broom death through shifts in the balance of growth regulators. Therefore, the factor does not directly affect the hosts hormonal balances. However, the toxin may in some way enhance the effectiveness of the host's own hormones, perhaps by altering membrane transport systems or by increasing the number of receptors in the cocoa cell membrane. This factor would be capable of producing distorted growth at low concentrations, while at higher concentrations capable of causing the death of host tissues. In addition, the factor would have to be relatively non-mobile in the host, because the host response to infection by *C. perniciosa*, broom formation, is relatively localised.

The distribution of *C. perniciosa* throughout a green broom

The results (Table 6.2) clearly show a steep gradient in chitin and hence *C. perniciosa* content along the length of the broom illustrated in Fig. 6.4 and 6.5. Scanning electron micrographs of the various sections taken along the broom are illustrated in Fig. 6.6-6.19. The vascular 'plug-like' tissue below the base of the broom had a significant concentration of *C. perniciosa* (66.55 µg/mg of dry sample weight SD 0.46). The vascular 'plug-like' tissue consisted of vascular tissue that was removed along with the broom proper, this tissue is labelled on Fig. 6.5. The results indicated that *C. perniciosa* hyphae had penetrated into the vascular tissue of the young cocoa plant's stem, and eventually the plant may have died. If *C. perniciosa* normally spreads away from the broom tissue then it may pose problems for the control of witches' broom through phytosanitation. Cronshaw & Evans (1978) also found *C. perniciosa* could spread some distance from the original infection point, they discovered that infected flowers could successfully produce beans which subsequently produced infected plants. However, these results do not contradict those from the IWBP (1991) which found that the only economically effective means of control for witches' broom was phytosanitation. But they do serve as a reminder that the fungus is probably capable of spreading a significant distance from the original infection point. When viewed under the SEM, the 'vascular-plug' tissue showed no clearly visible fungal hyphae. This was partly because of the nature of the tissue, which was in an advanced state of decomposition, and also because of the difficulty in seeing fungal hyphae in cocoa tissue unless in very high concentrations (Collin pers. comm.). It would probably have been possible to see hyphae at the concentration indicated by the chitin assay had the tissue not been so badly decomposed.

The next section was taken 1 cm from the 'vascular-plug' towards the meristem, this section constituted the base of the broom (Fig. 6.6). This section had extremely high concentrations of *C. perniciosa* (215.65 µg/mg of sample SD 0.46). The section when viewed under the SEM was clearly in an advanced stage of decomposition but a significant number of *C. perniciosa* hyphae were visible. The hyphae were thought to be secondary phase, in accordance with the observations made by Evans (1980), because both intra- and intercellular growth was visible. The section taken 1 cm from the base (Fig. 6.7) had a fungal concentration of 193.29 µg/mg of

dry sample weight (SD 0.46) it was also decomposed and necrotic. The SEM pictures of this section also showed the presence of high concentrations of *C. perniciosa* hyphae in the necrotic tissue. These hyphae were also seen growing both intra- and intercellularly, they were therefore considered to be secondary phase hyphae, in accordance with the observations made by Evans (1980).

The stem section taken 2 cm from the base (Fig. 6.8) contained considerably less *C. perniciosa* hyphae (68.05 μg/mg of dry sample weight SD 0.46), than the sections taken from the base and 1 cm from the base. The concentration of hyphae in the 2 cm from the base section was similar to that from the 'vascular-plug' tissue. Hyphae were clearly visible when the section was viewed under the SEM, with both intra- and intercellular hyphae present. The hyphae in this section were therefore considered to be secondary phase. The sections taken 3 and 4 cm from the base (Fig. 6.9 and 6.10) contained 39.80 μg/mg (SD 0.46) and 36.08 μg/mg of dry sample weight (SD 0.46) respectively. These sections therefore contained considerably less fungus than sections taken nearer to the base. When viewed under the SEM these two sections contained very few hyphae, too little to determine whether they were growing intra- or intercellularly. Both intra- and intercellular hyphae were seen in the 3 cm from the base section and were therefore considered to be secondary phase, but only intercellular hyphae were seen in the 4 cm from the base section, and were therefore possibly primary phase hyphae.

The bud taken 4 cm from the base contained 51.16 μg/mg of dry sample weight (SD 0.46) of *C. perniciosa*. The bud was beginning to burst and had a twisted appearance. When viewed under the SEM however, no fungal hyphae were seen.

The broom section taken 5 cm from the base (Fig. 6.11) contained only 6.62 μg/mg of dry sample weight (SD 0.46). This value was considerably lower than any of the values obtained for the sections taken between 4 cm from the base and the 'vascular-plug'. This section was the crossover point between the heavily infected necrotic region of the broom and the comparatively normal looking region of the broom. Only one *C. perniciosa* hypha was

observed when the section was viewed under the SEM. The hypha was growing intercellularly, and the cellular structure in that region appeared to be like normal cocoa tissue.

The fungal content of the broom section taken 6 cm from the base (Fig. 6.12) contained 11.40 μg/mg of dry sample weight (SD 0.46) of *C. perniciosa*. No fungal hyphae were seen when the section was observed under the SEM. The section taken 7 cm from the base, (Fig. 6.13) contained more *C. perniciosa*, the concentration was 24.30 μg/mg of dry sample weight (SD 0.46). This section carried a yellowing leaf and a necrotic petiole (see Fig. 6.5), which were not included in the chitin analysis procedure. When viewed under the SEM no fungal hyphae were seen. The next section, 8 cm from the base (Fig. 6.14), contained a *C. perniciosa* concentration of 20.22 μg/mg of dry sample weight (SD 0.46). This section contained a small necrotic patch. When viewed under the SEM, no fungal hyphae were seen and the tissue looked like normal cocoa stem tissue.

The sections taken from 9 to 12 cm from the base inclusive (Fig. 6.15-6.18), all contained low levels of *C. perniciosa* infection, ranging from 9-13 μg/mg of dry sample weight. No *C. perniciosa* hyphae were seen under the SEM, and the broom tissue looked like normal cocoa stem tissue.

The final 2 mm section of the broom stem, up to and including the meristem (Fig. 6.19), contained a *C. perniciosa* concentration of 24.09 μg/mg of dry sample weight (SD 0.46). This value was significantly higher than the previous 4 cm of broom stem and was approximately the same as the region encompassing 7-8 cm from the meristem. The region 7-8 cm from the meristem had visible signs of infection. The concentration of *C. perniciosa* in an infected bud taken 4 cm from the base was also similar to that found in the final broom section. The final section exhibited no visible signs of infection and when viewed under the SEM, no fungal hyphae were seen and the tissue appeared normal.

The fungal concentration varied considerably along the length of the broom. The precise variations in fungal distribution are unlikely to be mirrored in other brooms. However, a great deal of useful information was gained from the detailed data obtained from this broom. It was clear that the *C. perniciosa* hyphae were extremely localised within the broom, leaving the majority of the broom with only low levels of fungal infection. The hyphae tended to be localised at the base and in the 'vascular-plug' of the broom and also at the growing points. No other workers have analysed the distribution of *C. perniciosa* within a single broom in such detail. These results suggest that *C. perniciosa* can induce broom death even though the fungus itself is extremely localised within the broom tissue. In fact, the fungus appeared to be primarily localised to the vascular tissue. Earlier results indicated that the 'vascular-plug' tissue contained high levels of fungus. Because the hyphae appear to be present mainly at the base, they are capable of doing a great deal of damage by destroying the bases of stems, branches, and transport structures. Thus, comparatively little *C. perniciosa* biomass may be all that is required to kill a broom.

C. perniciosa hyphae also tended to be present at the growing points of the broom. The distorted growth originated from the growing points and *C. perniciosa* was present in high concentrations in this region. This feature lends further weight to the theory that *C. perniciosa* directly induces broom production rather than it being a systemic response to infection.

If a broom does not 'grow-through', it will die and eventually dry out. *C. perniciosa* is known to prefer dry conditions (Bravo-Velasquez & Hedger, 1988) and to have a competitive advantage over other saprotrophic fungi in relatively dry substrates (Bravo-Velasquez & Hedger, 1988). Therefore, any action which reduces the transpiration stream and thereby causes broom drying will ensure that *C. perniciosa* has a competitive advantage over other potential saprotrophs. In addition, results from the IWBP (1991) demonstrated that *C. perniciosa* produced more basidiomes from brooms with a low water content than from wetter brooms.

In the light of this new information, a few additions can be proposed to the accepted life style of *C. perniciosa*. There is circumstantial evidence for the following suggestions, these are; the initial infection process is limited to the growing points of the tree. The fungus grows biotrophically and therefore induces broom formation to ensure an expanding food source. Broom formation is induced by a non-translocatable toxin which at low concentrations distorts growth but at high concentrations causes necrosis. The toxin is non-translocatable and therefore broom production is localised. The fungus is concentrated at the base of the broom and a developing ring of necrotic tissue disrupts water transport, thereby inducing broom drying. The secondary phase of the fungus, which is well adapted to a drier environment, then becomes dominant and eventually sporulates. The drier environment ensures competition from other saprotrophs is minimised.

6.1.1d Conclusions

The chitin content varied over time and between the primary and secondary phases of *C. perniciosa*. The mean concentration of chitin in the primary phase of *C. perniciosa* was 14.40 µg/mg of dry fungal weight (SD 4.90) while in the secondary it was 19.48 µg/mg of dry fungal weight SD (9.20). The mean concentration of chitin in *C. perniciosa* taken for both the primary and secondary phases was 16.36 µg/mg of dry fungal weight (SD 7.55). Thus the corresponding conversion factor of chitin content to fungal content was calculated as 61.12 (SD 28.32). The *C. perniciosa* estimation procedure based upon the new chitin assay is an extremely useful tool for investigating fungal invasion in cocoa tissues.

High levels of chitin and therefore *C. perniciosa* hyphae were present in both green and brown brooms. The concentration in green brooms was 81.48 µg/mg of dry sample weight (SD 0.46) and 160.97 µg/mg of dry sample weight (SD 0.46) in the brown brooms. The high concentrations of *C. perniciosa* in both green and brown brooms indicated broom formation was linked to the direct presence of the fungus in the broom tissue. Thus, broom formation was directly associated with *C. perniciosa* hyphae rather than being produced as a systemic response to infection by the fungus.

The death of green brooms and the formation of the brown brooms was probably not due to the increase in fungal biomass associated with the brown brooms; the difference in fungal biomass between the two types of broom was almost certainly insufficient. Another factor probably causes the death of the green brooms. This factor is potentially a toxin which induces distorted growth at low concentrations and tissue death at higher concentrations.

C. perniciosa concentrations varied considerably along the length of the broom analysed in detail, section by section. The most fungus was found in the basal 2 cm of the broom which contained approximately 200 μg/mg of dry sample weight. Very high concentrations of fungus were also found in the region 2-4 cm from the base. The concentration of *C. perniciosa* was much lower up to the region 7-8 cm from the base. This region, 7-8 cm from the base, contained increased levels of *C. perniciosa*, of approximately 20 μg/mg of dry sample weight respectively, visible signs of infection were also present. The fungal concentration was less below the meristematic region. The fungal concentration in the meristematic region, 1 cm up to and including the meristem, contained 24.09 μg/mg of dry sample weight (SD 0.46). A bud 4 cm from the base also contained relatively high levels of *C. perniciosa*, the concentration was 51.16 μg/mg of dry sample weight (SD 0.46). The fungus was therefore concentrated at the base and at the growing points of the broom.

Fig. 6.1. Variation of the chitin concentration with time in the primary phase of *C. perniciosa*. For full details see 6.1.1.

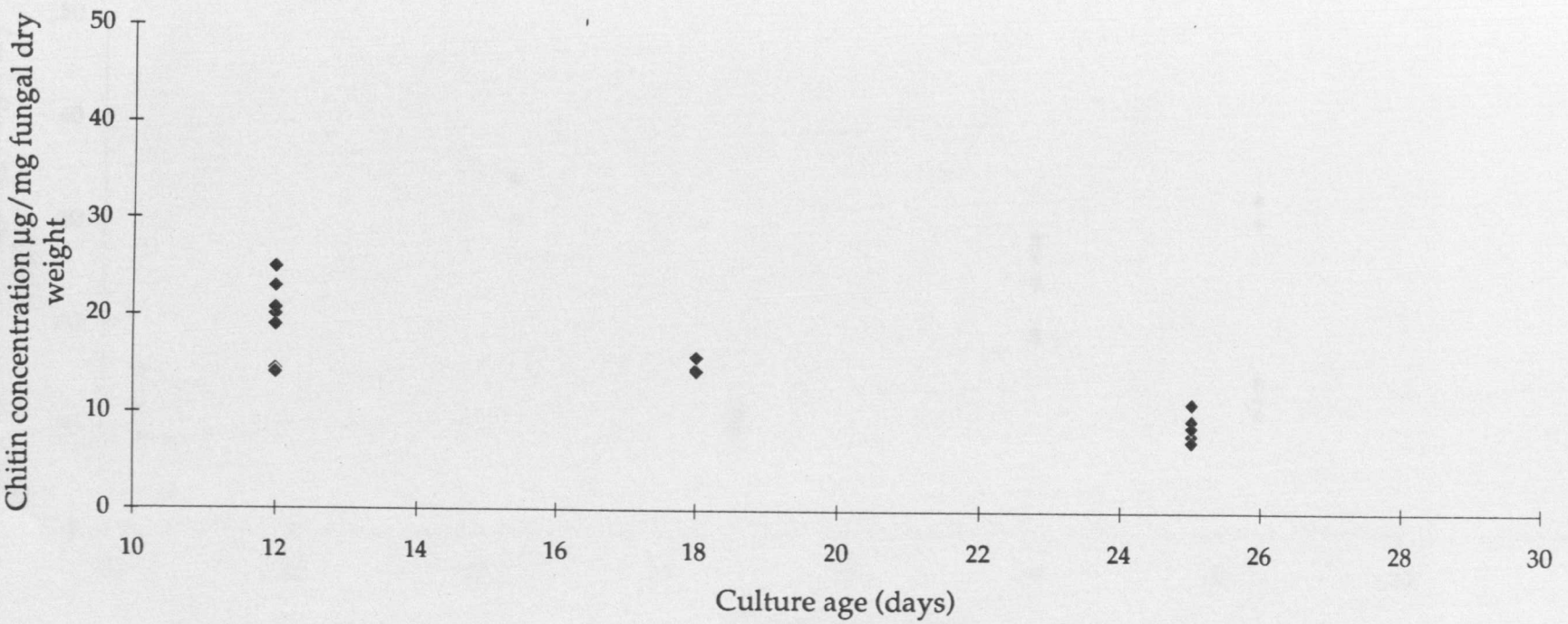


Fig. 6.2. Variation of the chitin concentration with time in the secondary phase of *C. perniciosa*. For full details see 6.1.1.

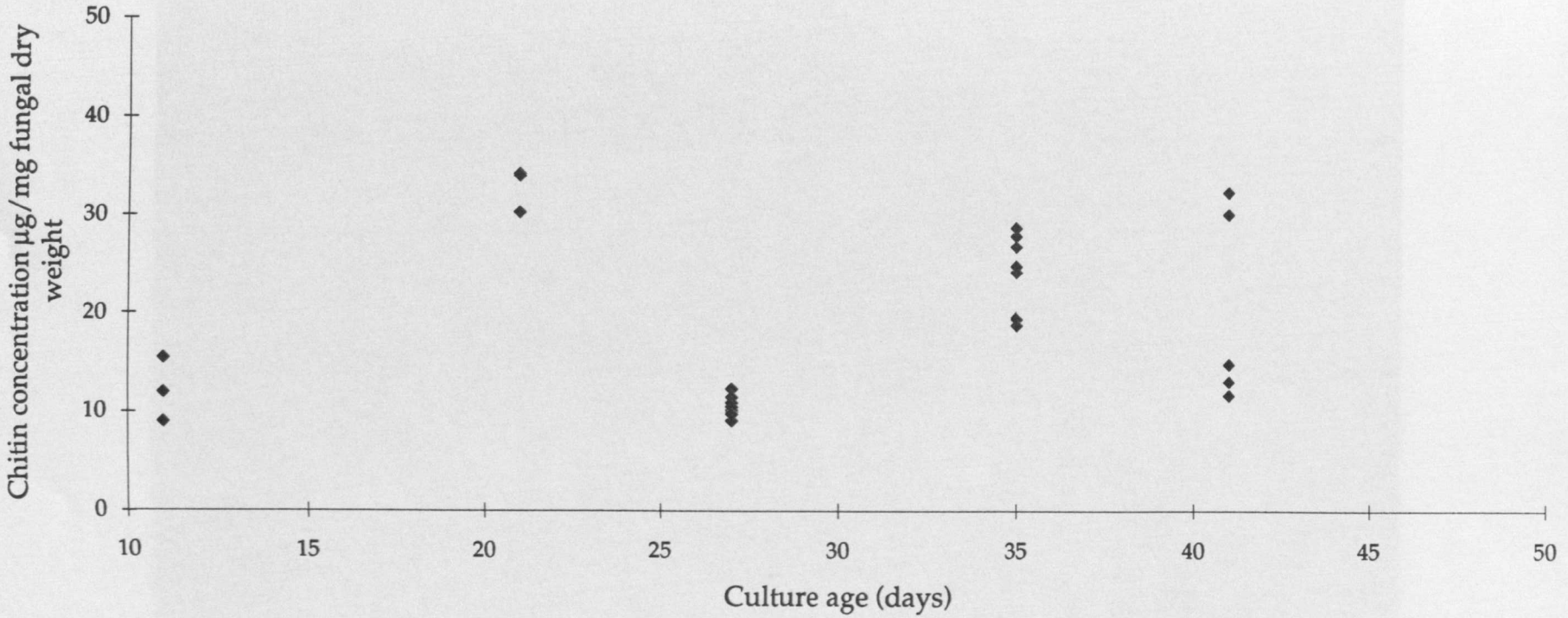


Fig. 6.3. A representative witches' broom.

Fig. 6.4. The intact cocoa plant before removal of the witches' broom (WB) used in experiment 6.1.1.

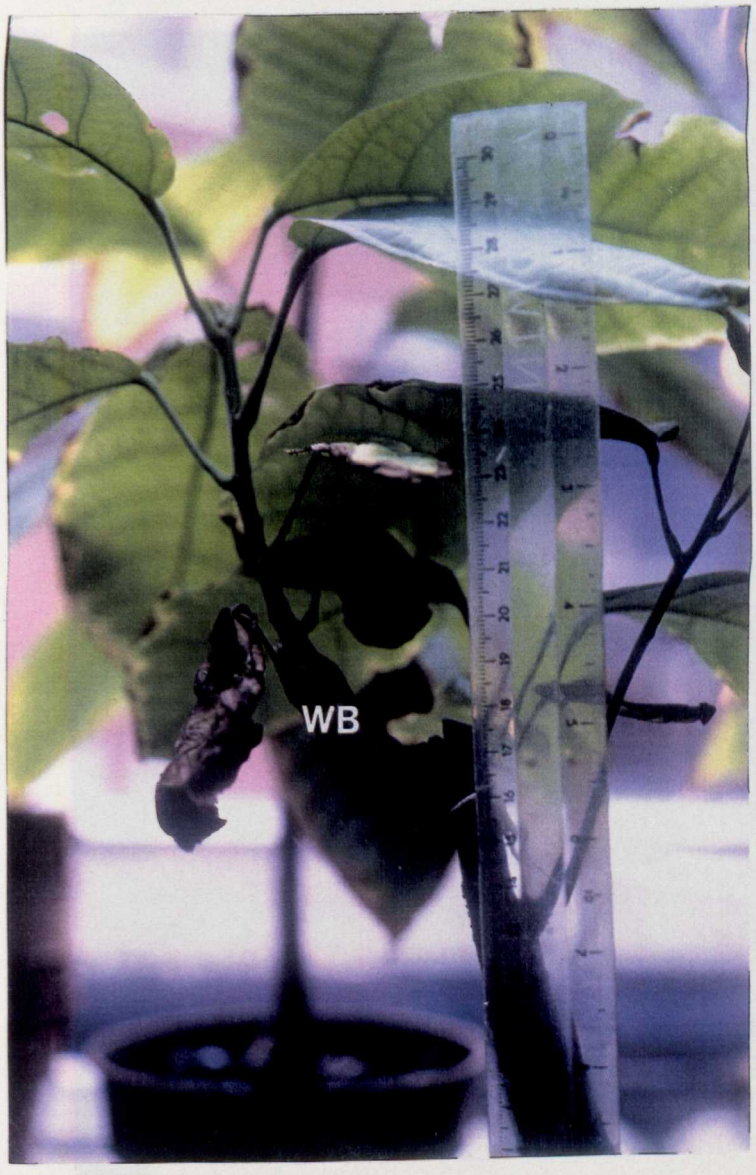


Fig. 6.5. The witches' broom used in experiment 6.1.1 after removal from the host cocoa plant. The base of the broom (B) and the 'vascular-plug' (V) are indicated on the photograph.

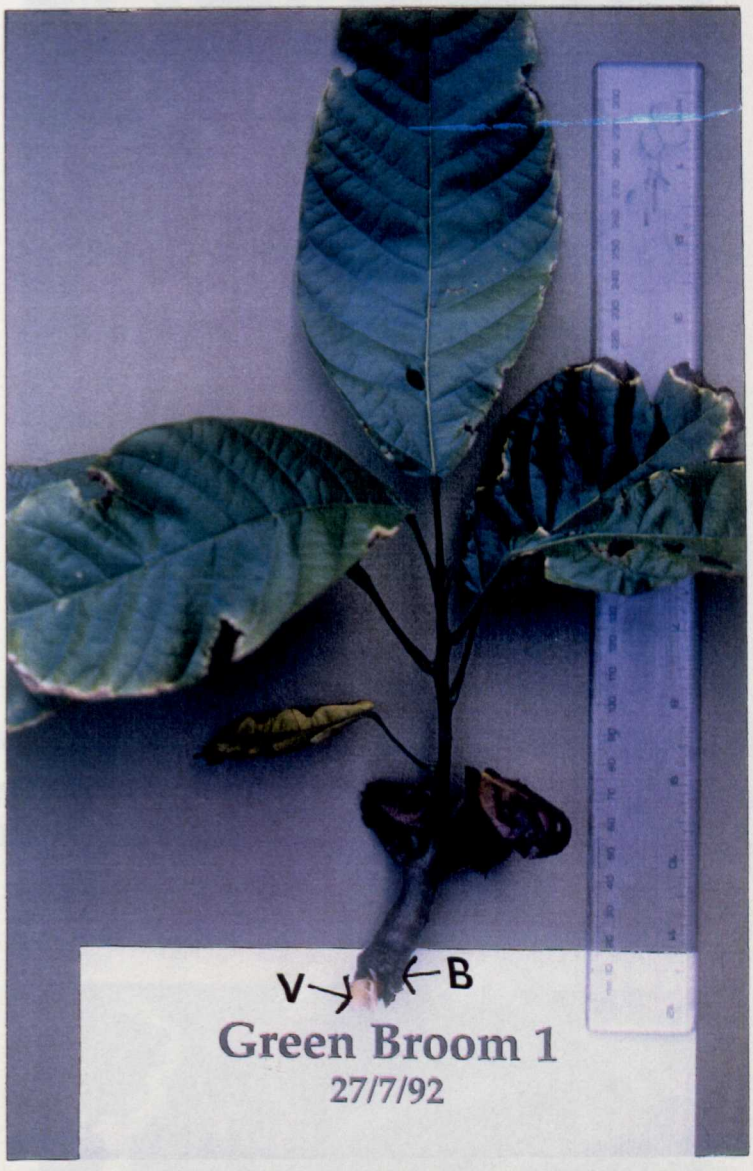


Fig. 6.6. Scanning electron micrograph of a section taken from the base of the single green broom analysed in detail in 6.1.1. Hyphae (H) were seen in both the intercellular spaces (IS) and the plant cells (PC). Scale bar units equal 10 µm.

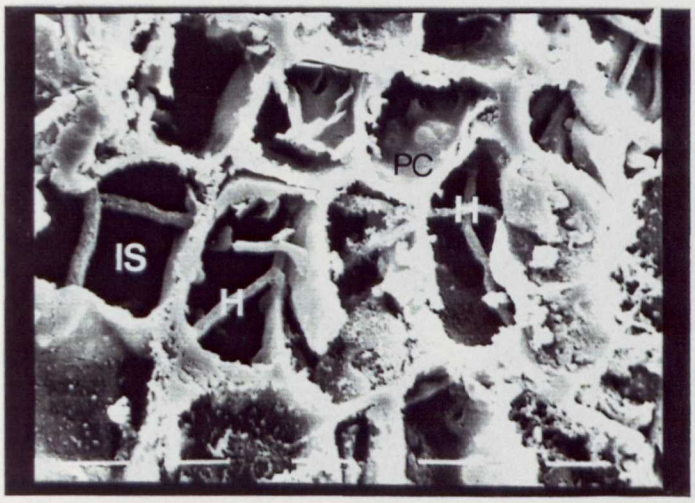


Fig. 6.7. Scanning electron micrograph of a section taken 1 cm from the base of the single green broom analysed in detail in 6.1.1. Hyphae (H) were seen in both the intercellular spaces (IS) and the plant cells (PC). Scale bar units equal 10 µm.

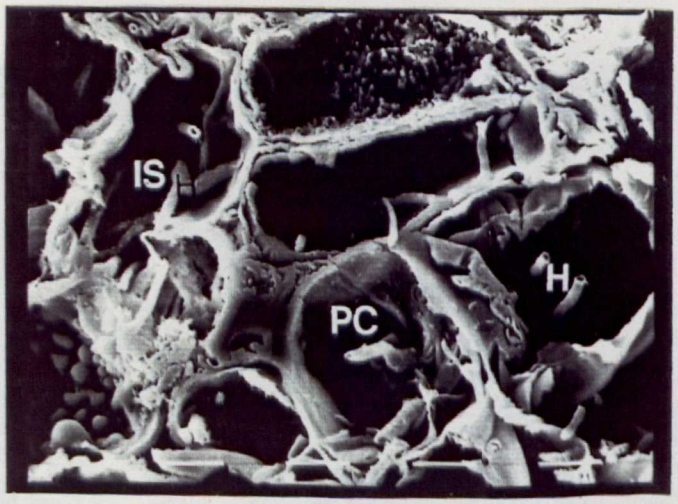


Fig. 6.8. Scanning electron micrograph of a section taken 2 cm from the base of the single green broom analysed in detail in 6.1.1. Hyphae (H) were seen in both the intercellular spaces (IS) and the plant cells (PC). Scale bar units equal 1 μm.

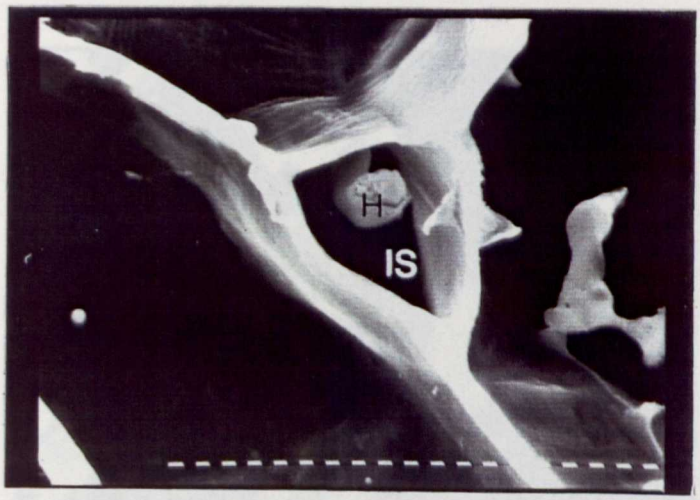


Fig. 6.9. Scanning electron micrograph of a section taken 3 cm from the base of the single green broom analysed in detail in 6.1.1. Hyphae (H) were seen in both the intercellular spaces (IS) and the plant cells (PC). Note the swollen and distorted hyphae (H) representative of primary phase *C. perniciosa* (Pegus, 1972; Evans, 1980). Scale bar units equal 1.3 μm.

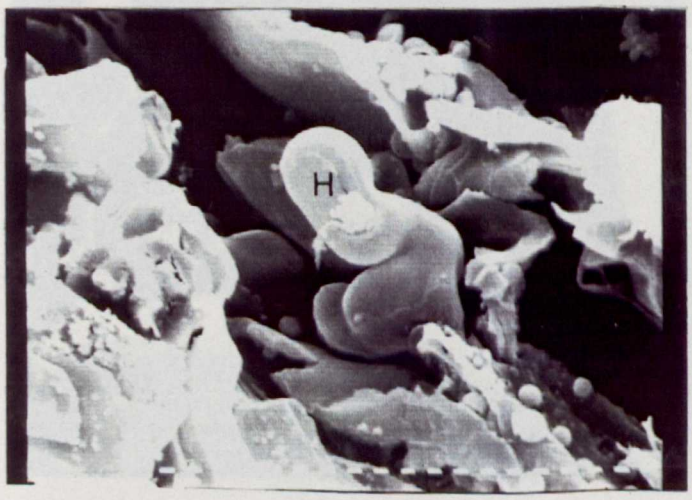


Fig. 6.10. Scanning electron micrograph of a section taken 4 cm from the base of the single green broom analysed in detail in 6.1.1. Hyphae (H) were seen in only the intercellular spaces (IS). Note the swollen and distorted hyphae (H) representative of primary phase *C. perniciosa* (Pegus, 1972; Evans, 1980). Scale bar units equal 1 μm.

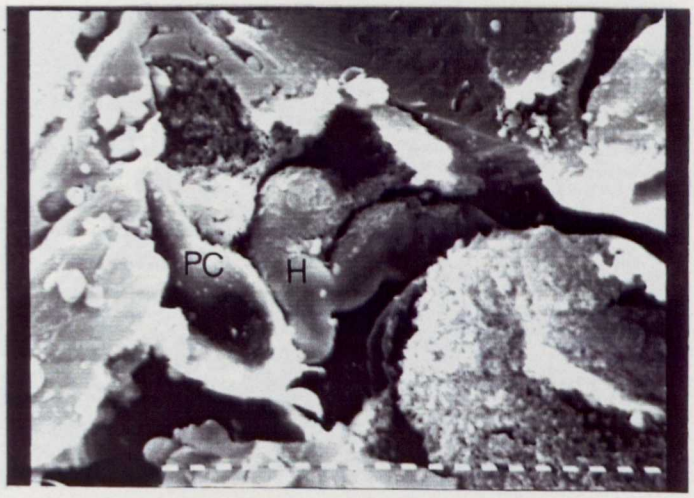


Fig. 6.11. Scanning electron micrograph of a section taken 5 cm from the base of the single green broom analysed in detail in 6.1.1. Only one hypha was seen (H) and was growing in the intercellular spaces (IS). The hypha was not swollen and distorted and was therefore representative of the secondary phase of *C. perniciosa* (Pegus, 1972; Evans, 1980). Scale bar units equal 1 μm.

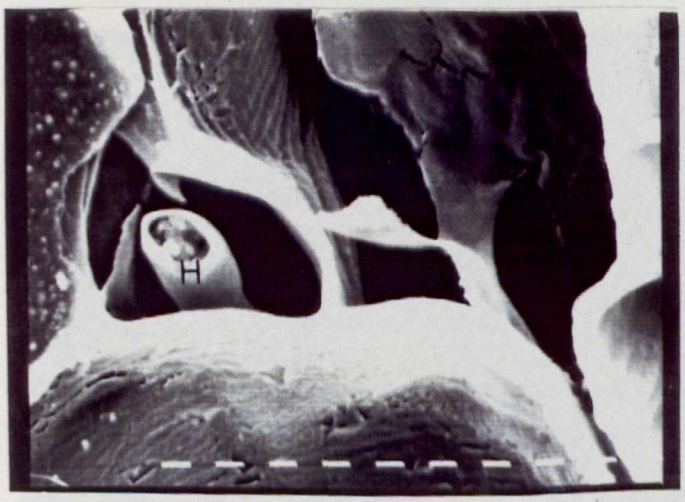


Fig. 6.12. Scanning electron micrograph of a section taken 6 cm from the base of the single green broom analysed in detail in 6.1.1. No hyphae were visible. Cocoa tissues identified were as follows; cortex (C), phloem (PH), cambium (CA), and xylem (X). Scale bar units equal 100 µm.

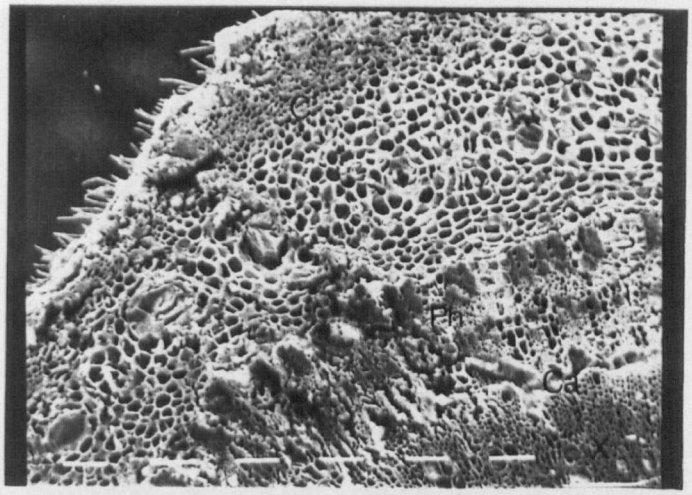


Fig. 6.13. Scanning electron micrograph of a section taken 7 cm from the base of the single green broom analysed in detail in 6.1.1. No hyphae were visible. Cocoa tissues identified were as follows; cortex (C), phloem (PH), cambium (CA), xylem (X), and pith (P). Scale bar units equal 100 µm.

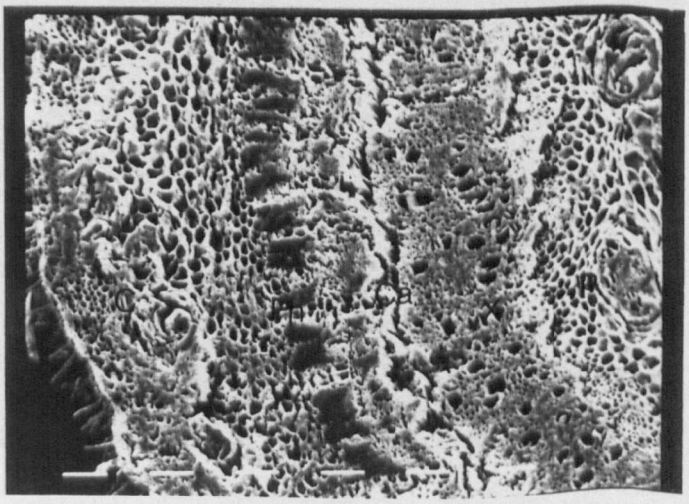


Fig. 6.14. Scanning electron micrograph of a section taken 8 cm from the base of the single green broom analysed in detail in 6.1.1. No hyphae were visible. Cocoa tissues identified were as follows; cortex (C), phloem (PH), cambium (CA), xylem (X), and pith (P). Scale bar units equal 100 μm.

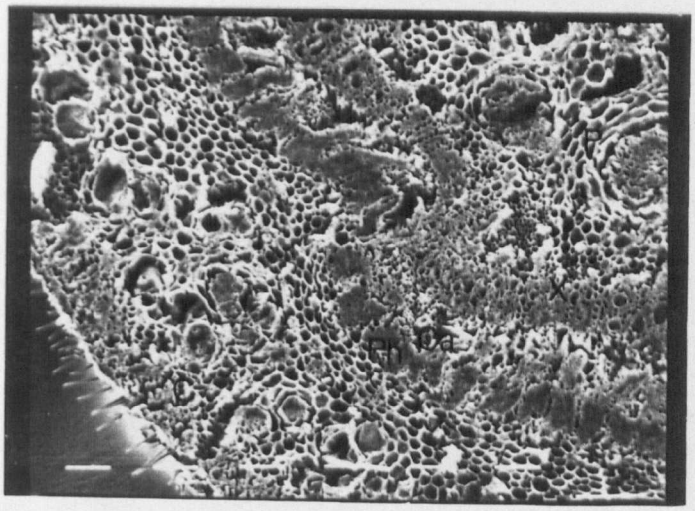


Fig. 6.15. Scanning electron micrograph of a section taken 9 cm from the base of the single green broom analysed in 6.1.1. No hyphae were visible. Cocoa tissues identified were as follows; cortex (C), phloem (PH), and xylem (X). Scale bar units equal 100 μm.

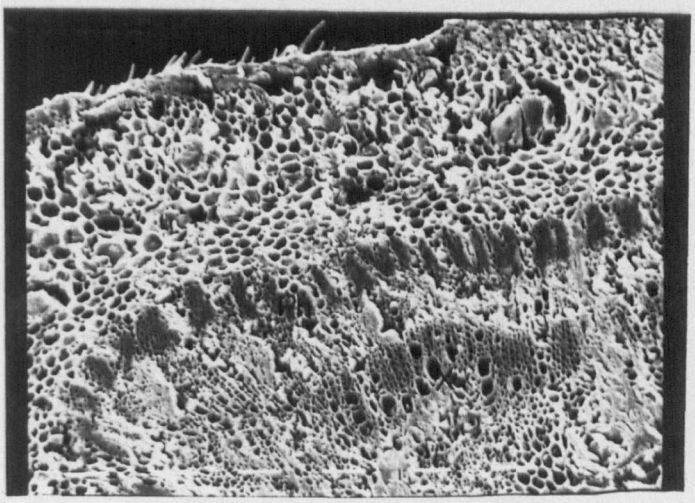


Fig. 6.16. Scanning electron micrograph of a section taken 10 cm from the base of the single green broom analysed in detail in 6.1.1. No hyphae were visible. Cocoa tissues identified were as follows; cortex (C), phloem (PH), cambium (CA), and pith (P). Scale bar units equal 100 μm.

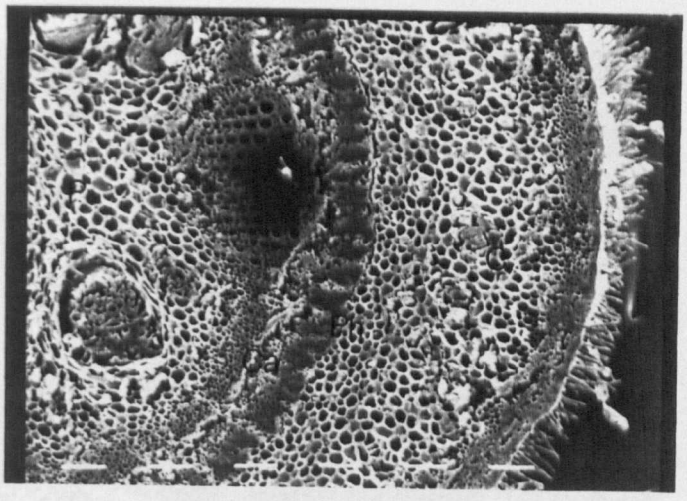


Fig. 6.17. Scanning electron micrograph of a section taken 11 cm from the base of the single green broom analysed in detail in 6.1.1. No hyphae were visible. Cocoa tissues identified were as follows; cortex (C), phloem (PH), and xylem (X). Scale bar units equal 100 μm.

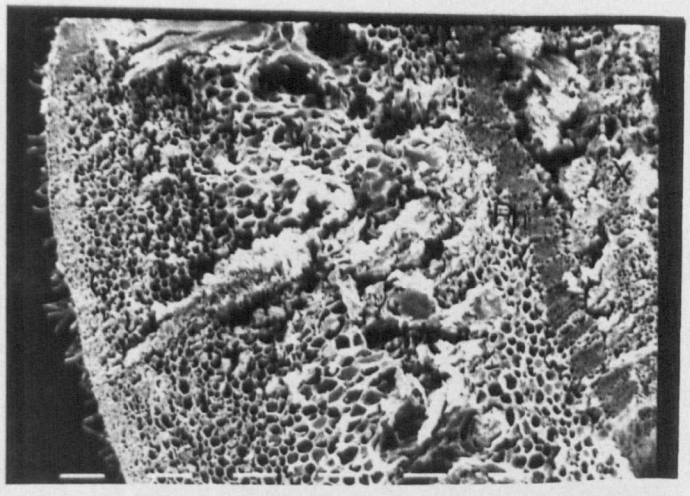


Fig. 6.18. Scanning electron micrograph of a section taken 12 cm from the base of the single green broom analysed in detail in 6.1.1. No hyphae were visible. Cocoa tissues identified were as follows; cortex (C), phloem (PH), cambium (CA), xylem (X), and pith (P). Scale bar units equal 100 μm.

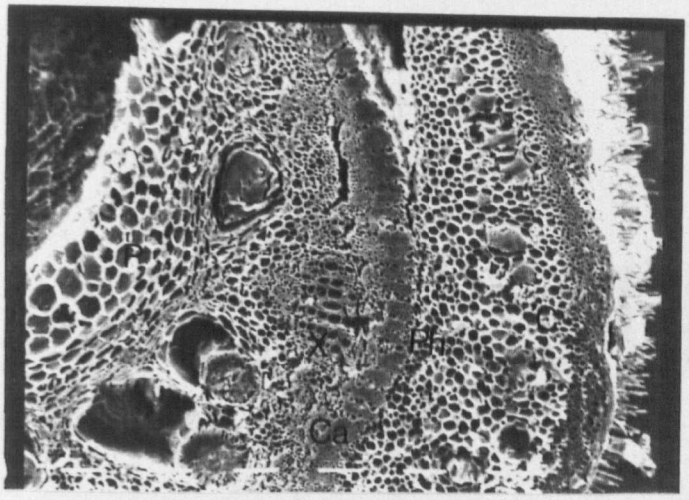


Fig. 6.19. Scanning electron micrograph of a section taken 13 cm from the base of the single green broom analysed in detail in 6.1.1. No hyphae were visible. Only pith tissue (P) was identified. Scale bar units equal 10 μm.

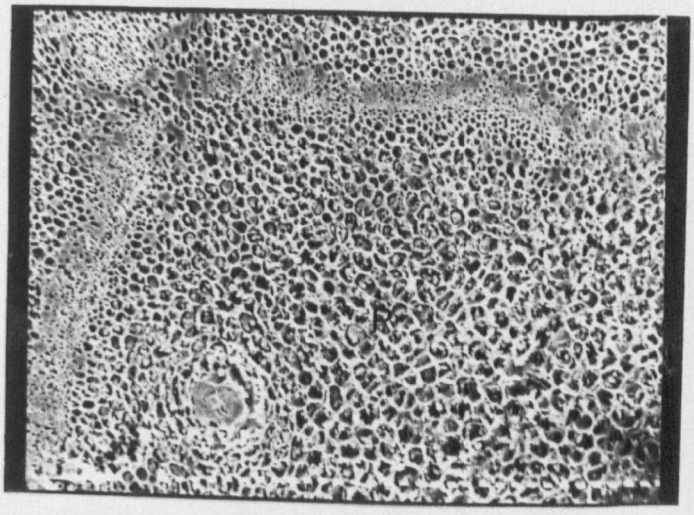


Table 6.1. The concentration of chitin and *C. perniciosa* in green and brown brooms.

	Concentration of chitin µg/mg dry cocoa tissue (standard deviation)	Concentration of *C. perniciosa* µg/mg dry cocoa tissue (standard deviation)
Green broom 1	1.39 (0.14)	85.14 (0.47)
Green broom 2	1.15 (0.21)	70.19 (0.50)
Green broom 3	1.39 (0.12)	85.17 (0.47)
Green broom 4	1.26 (0.05)	77.16 (0.47)
Green broom 5	1.49 (0.08)	90.98 (0.47)
Average for green brooms	1.33 (0.17)	81.48 (0.46)
Brown broom 1	2.98 (0.20)	182.33 (0.47)
Brown broom 2	3.24 (0.11)	198.14 (0.46)
Brown broom 3	2.62 (0.17)	160.23 (0.47)
Brown broom 4	2.54 (0.33)	155.07 (0.48)
Brown broom 5	2.14 (0.13)	128.58 (0.47)
Average for brown brooms	2.63 (0.43)	160.97 (0.46)

Table 6.2. The concentration of chitin and *C. perniciosa* in transverse sections taken every centimetre along the length of the single green broom described in the main text.

Description of section	Chitin concentration µg/mg dry cocoa tissue (standard deviation)	Concentration of *C. perniciosa* µg/mg dry cocoa tissue (standard deviation)
Basal vascular tissue	1.09 (0.06)	66.55 (0.46)
Base	3.53 (0)	215.65 (0.46)
1 cm from base	3.16 (0.17)	193.29 (0.46)
2 cm from base	1.11 (0.08)	68.05 (0.46)
3 cm from base	0.65 (0.04)	39.80 (0.46)
4 cm from base	0.59 (0.04)	36.08 (0.46)
5 cm from base	0.11 (0.01)	6.62 (0.46)
6 cm from base	0.19 (0.01)	11.40 (0.46)
7 cm from base	0.40 (0.03)	24.30 (0.46)
8 cm from base	0.33 (0.03)	20.22 (0.46)
9 cm from base	0.15 (0.01)	9.36 (0.46)
10 cm from base	0.15 (0.01)	9.14 (0.46)
11 cm from base	0.20 (0.003)	12.41 (0.46)
12 cm from base	0.15 (0.15)	9.62 (0.46)
13 cm from base	0.39 (0.03)	24.09 (0.46)
Infected basal bud	0.84 (0.01)	51.16 (0.46)

Chapter 7

The molecular biology of *Crinipellis perniciosa*

7.1.1a Introduction

Little, if anything, is known about the molecular biology of *C. perniciosa*. Equally, very little is known about the range of different pathotypes of *C. perniciosa*, although Hedger *et al.* (1987) and latterly McGeary & Wheeler (1988) have begun the process of classifying *C. perniciosa* isolates into compatibility groups. Ultimately, *C. perniciosa's* pathogenicity depends upon the genetic constitution of the fungus. Information about fungal genetics has traditionally relied upon compatibility testing and Mendelian genetics. But in recent years, molecular biology has allowed investigation of the pathogenicities of a number of fungal species.

It would be useful to know the relationship between different pathogenic groups of *C. perniciosa* isolates in relation to susceptible cocoa cultivars. It is not unreasonable to assume that a few cocoa cultivars are resistant to certain *C. perniciosa* isolates. For example, the cocoa cultivar Scavina 6, widely grown in Trinidad & Tobago, was resistant to certain isolates of *C. perniciosa*. Unfortunately, this resistance has since 'broken-down'. The planned cocoa resistance breeding programme would be greatly aided by a simple and rapid method of assessing the relative pathogenicity of a *C. perniciosa* isolate in relation to a cocoa cultivar. For example, cocoa cultivars of known resistance to different pathogenic groups of *C. perniciosa* could be crossed to produce resistance to more or all pathogenic groups of *C. perniciosa*. It is also possible that a cocoa cultivar resistant to *C. perniciosa* in one area may be identified and grown in other regions where it may also be resistant to the endemic population of *C. perniciosa*.

A method which allowed the rapid and accurate testing of pathogenicity in *C. perniciosa* would also be of direct use in controlling witches' broom disease. There is a great deal of pathogenic variability amongst *C. perniciosa* isolates (Wheeler pers. comm.). Some *C. perniciosa* strains are likely to be considerably less pathogenic than others, with some strains possibly being wholly biotrophic or even non-pathogenic. If certain strains are non-pathogenic, then potentially they could be used as fungal epiphytes. If a wholly biotrophic strain could be isolated then potentially it may be capable of out-competing the more pathogenic strains, and thus the impact of witches' broom disease would be lessened.

Leptosphaeria maculans (Dem) Ces. et de Not., the sexual stage of *Phoma lingam* (Tode) Desm., is a pathogen of many crucifers, most notably *Brassica napus* L. (rape seed). This pathogen has two stages of growth. The first stage is essentially biotrophic and the second stage is saprotrophic (Hammond & Lewis, 1987). There are two pathogenesis groups of *L. maculans*. One group is pathogenic whilst the other is non-pathogenic. A rapid method of identifying the pathogenic group of a pathotype isolated from the field was required. Schäfer & Wöstemeyer (1992) developed a rapid method of assessing pathogenicity in *L. maculans* using a random primer dependent polymerase chain reaction test (RAPD PCR). RAPD PCR is pronounced rapid PCR.

The basis of PCR is relatively simple. The target DNA is first heat denatured to produce single stranded DNA. Then a primer consisting of complementary bases to the target sequence is annealed onto the gene to be amplified. Taq polymerase then proceeds to polymerise or elongate the single stranded DNA, beginning at the primers. The DNA is elongated until either the temperature is raised sufficiently to prevent further polymerisation, or is physically prevented by the presence of another primer sequence. The amplification step is typically repeated 30-40 times so that the DNA sequences are amplified many millions of times (Schäfer & Wöstemeyer, 1992).

Until recently, DNA amplification was confined to relatively well mapped regions of the genome. Using conventional PCR techniques, a degree of sequence information is required to enable the construction of complementary primers. Thus, PCR was confined to a relatively few well known and therefore well characterised genes. The major breakthrough occurred with RAPD PCR. The point of difference between PCR and RAPD PCR is the use of random primers, hence Random Amplified Polymorphic DNA (Schäfer & Wöstemeyer, 1992).

These primers anneal specifically but randomly throughout the genome. The annealing is random only in the sense that the exact target destination of the genome is unknown, the annealing is still consistent for each amplification step. If a complementary sequence to the primer is present, then the sequence will be amplified in all isolates and in all species. But if

the sequence is not, or differs by only one or two bases then the sequence will not be amplified. Thus, minute pathotype specific differences can be distinguished. The use of random primers allows DNA amplification even if no sequence information is available. When the amplified DNA is separated using polyacrylamide gel electrophoresis characteristic banding patterns are produced. These patterns are specific in two ways. Firstly, they depend on the genetic constitution of the strain, to such a degree that single base changes in DNA may be detected in some instances (Williams *et al.*, 1990). Secondly, they depend on the initial primer sequence, primers can therefore be 'fine-tuned' in order to emphasise differences in the genomic DNA between strains (Schäfer pers. comm., Institut Für Genbiologische Forschung (IGF), Berlin).

Schäfer & Wöstemeyer (1992) developed the test for pathogenicity in *L. maculans* at the IGF, Berlin. It was decided to use their RAPD PCR method of pathogenicity testing on *C. perniciosa*. The work for this chapter was carried out with the kind help and support of Christine Schäfer and Johannes Wöstemeyer at IGF, Berlin. The method was adapted for the Manaus F strain, with the aim of developing the method for others to use with *C. perniciosa*. Time constraints limited the testing to one strain, but there is no reason to suppose that it cannot be used for the large scale screening of different *C. perniciosa* isolates. Any modification of the primer sequence necessary to emphasise strain specific differences can be done once the technique has been mastered.

7.1.1b Methods

Approximately 10 mg of samples of primary and secondary phase *C. perniciosa* were ground under liquid nitrogen in a mortar and pestle. The resulting powders were then transferred to microfuge tubes. Aliquots (1 ml) of 1 % SDS in 10 mM EDTA were then added to inhibit DNAases. The samples were then vortex mixed for 1 min.

The proteins were precipitated from the samples upon the addition of 70 mg of NaCl. The samples were then left in an ice bath for 30 min. The samples were centrifuged in a microfuge for 10 min at 12000 rpm, and 1 ml of the DNA containing supernatants were transferred to

fresh microfuge tubes. PEG 6000 (100 mg) was added to the samples, the DNA was precipitated when the samples were shaken. The samples were then left in an ice bath for 1 hr, and then centrifuged at 12000 rpm for 15 min.

The supernatants were removed and discarded. The DNA containing pellets were then re-dissolved in 180 µl of TE buffer (10 mM tris-borate buffer with 0.1 mM EDTA). Twenty µl of 4M LiCl was added to precipitate the DNA, then 200 µl of iso-propanol was added. The samples were transferred to an ice bath for 30 min then centrifuged for 15 min at 12000 rpm. The supernatants were removed and discarded. Fifty µl of 70 % ethanol was added and the samples vortexed. The samples were once again centrifuged at 12000 rpm for 5 min, and the supernatants removed. The pellets were dried in a vacuum desiccator.

The resulting stock DNA samples were re-dissolved in 30 µl of TE buffer. Aliquots (5 µl) were removed from the stock DNA solutions and mixed with 5 µl of water and 3 µl of loading buffer (see below). The resulting mixtures were loaded on to a minigel, together with kilobase markers ranging in size from 75 - 12216 bases. The gels were run at 60 V for approximately 2 hr in a solution of tris-borate buffer. The bands on the minigel were developed by immersing in a solution of 1.3 µg/ml of ethidium bromide for approximately 30 min. The gels were washed 5 times in distilled water and photographed under UV light using a transilluminator.

Aliquots (10 µl) of the original stock DNA solutions were diluted with 90 µl of distilled water, 5 µl of the resulting solutions were transferred to microfuge tubes. Five µl of Taq polymerase buffer (supplied with enzyme), 4 µl of 2.5 mM dNTP (consisting of 2.5 mM each of dATP, dGTP, dCTP, dTTP), 3 µl of 50 mM magnesium acetate, 0.5 µl of Taq polymerase solution containing 2.5 units, and 28.5 µl of redistilled water, were added to each microfuge tube. Aliquots (4 µl) containing 40 ng of the appropriate primer was then added to each solution. The final volume of the solutions was 50 µl. The solutions were then overlaid with 50 µl of highly liquid paraffin.

The PCR amplification cycle was; 95 °C for 20 sec, 32 °C for 60 sec, and 72 °C for 20 sec. The cycle was repeated 28 times. The amplificates were then heated at 72 °C for 6 min.

The samples were then removed from under the paraffin and reduced in volume to about 30 µl in a Speedivac concentrator. Aliquots (4 µl) of loading buffer (see below) were then added to the samples which were then loaded on to polyacrylamide gels. Molecular weight marker solutions with fragments ranging in size from 75 - 12216 bases were added to the end lanes of the gels. The gels were then run at 60 V in a solution of tris-borate buffer for approximately 6 hr. The bands on the gel were developed using a solution of 1.3 µg/ml ethidium bromide. After approximately 30 min the gels were washed 5 times in redistilled water then viewed and photographed using a UV transilluminator.

Primer sequences used

Primer code number	Primer sequence
3	5' ACGGTCTTGG 3'
4	5' GCCGTAGTAC 3'
5	5' ACGATCGCGG 3'
6	5' GAAACAGCGG 3'
8	5' GGAGCCCAC 3'
11	5' TGGGTCTAGT 3'
14	5' GCCGTCTACG 3'
16	5' CAATGGAGTG 3'
17	5' GGCATCGGCC 3'
21	5' GTGAGCGTC 3'

Primer production

Primer production was achieved using an Applied Biosystems automatic oligonucleotide synthesiser with reagents and conditions as recommended by the manufacturer.

Loading buffer

The stock loading buffer consisted of 50 mg bromophenol blue, 50 mg xylenecyanol, 1.5 ml glycerine, and 100 µl 0.5 M EDTA. The solution was then made up to 5 ml with redistilled water. Before use, the loading buffer was diluted down to a 10 % solution with a diluting solution consisting of 10 mM EDTA, 30 % glycerine, and 70 % redistilled water.

Gel production

The minigels and the main gels consisted of 0.8 % and 1.2 % agarose respectively in TBE buffer. The TBE buffer consisted of 0.089 M tris-borate buffer and 0.002 M EDTA.

Suppliers

Bromophenol blue, tris-borate buffer, and EDTA were supplied by Sigma Chemical Co. Ltd., Taq polymerase, and dNTP's by Boehringer Mannheim, xylenecyanol, highly liquid paraffin, and Peg 6000 (polyethylene glycol) by Merck, agarose by SeaKem Ltd, kilobase markers by Gibco BRL, electrophoresis tanks by BioTee Fischer Ltd., with power units by Bio-Rad Ltd., Microfuge, model Biofuge A by Victor Reckor, Genie 2 vortex mixer by Bender and Hobein AG, Polaroid film and camera supplied by Polaroid Ltd., UV transilluminator by Bachofer, Speedivac concentrator, model SVC 100H, by Savant Ltd., Trio Thermoblock PCR cycle block supplied by Biometra.

7.1.1c Results and Discussion

Fig. 7.1 clearly illustrated that the DNA preparation was successful. The bands shown in lanes a, b, and c were obtained from primary phase DNA extracts, while those in lanes d, e and f were obtained from secondary phase DNA extracts. The gel also indicated that the quantities of DNA extracted were roughly the same for all the samples extracted. The minigel strongly indicated that the primary phase contained fungal plasmids while the secondary phase did not (Hänfler pers. comm., IGF, Berlin). Fungal plasmids were indicated on the minigel by the presence of a faint secondary band beneath the main band (Hänfler pers. comm.) The plasmid band is indicated on Fig. 7.1. The plasmid band was present in lanes a and b but not in lanes d, e, and f, lane c presented an ambiguous result. Absolute

confirmation of the presence of plasmids in the primary phase would have required re-running the DNA samples after treatment with a restriction endonuclease. If the plasmid bands were no longer present after such treatment, then the faint secondary DNA band would have been due to plasmid DNA. Unfortunately, this extra experiment could not be performed because of time constraints.

Plasmids are relatively rare in eukaryotes. Sinclair *et al.* (1967) discovered the first example, the so called 2 μm DNA from *Saccharomyces cerevisiae.* Derivatives of this plasmid are widely used as transformation tools in baker's yeast. Stahl *et al.* (1978) discovered the first plasmid in filamentous fungi. Most plasmids in fungi are associated with mitochondria (Hänfler *et al.*, 1992). Few fungal plasmids are associated with traits conferring obvious selective advantages, most appear to code simply for their own replication (Hänfler *et al.*, 1992). Although some fungal plasmids are thought to code for aggressivity in plant pathogens (Hänfler *et al.*, 1992).

If plasmids are present in the primary phase but not in the secondary then clearly they must go somewhere. Two possibilities exist. The first is that they are destroyed by the fungus. This is extremely unlikely, it is difficult to envisage such a situation existing for longer than a few generations unless a strong selective advantage existed. The most likely possibility is that the plasmid becomes incorporated into the genomic or mitochondrial DNA. A possible scenario is that the plasmid codes for certain essential biotrophic functions which are not required in the secondary phase. The plasmid could therefore serve to increase the gene copy number or simply allow for easier expression. The plasmid may then become re-incorporated into the genome. At some stage, either at spore formation, or during germination, the plasmid forming DNA would leave the genome, circularise, and become a plasmid again.

The most exciting rôle for plasmids in *C. perniciosa* would be as agents of aggressivity or pathogenicity. If plasmids are associated with the most or least aggressive strains of *C. perniciosa*, then potentially, isolates could be grouped on the basis of presence or absence of plasmids. Cases of witches' broom could then be rapidly assessed for the likely impact on the

plantation or the cocoa growing region. Appropriate counter-measures could then be employed. With such a scenario, only a total DNA extract taking a few hours would be required to indicate the pathogenic group of the strain.

The results shown in Figs. 7.2 - 7.5 indicated clearly that only 1 primer, primer number 5, proved to be successful at promoting amplification in all samples. Primers 3, 6, and 8, worked for only 1 sample of the primary phase. Primer number 21 promoted amplification for one sample of the primary phase and for one sample of the secondary phase. Primers 4, 11, 14, 16, and 17, did not promote any amplification.

Thus, the RAPD PCR technique was successfully adapted for use in *C. perniciosa*. Primer number 5 allowed amplification, but it may not have allowed the differentiation between species, or between different pathogenic groups of *C. perniciosa*. Thus, the next stage would be to test the procedure on different pathogenic groups of *C. perniciosa*. The work may then have to concentrate on using different random primers to differentiate between pathogenic groups of *C. perniciosa*. The next stage of the work would be largely a matter of trial and error using different random primers to highlight pathogenic differences between strains and pathotypes.

However, there are certain features within the group of primers promoting even limited amplification that could serve as a guide to selecting appropriate primers for testing. All primers allowing amplification in at least one sample began with an A or a G at the 5' end, and ended with a G or a C at the 3' end. Nonamers and decamers were equally represented. The G+C content was at least 60 % with primer number 5 having a G+C content of 70 %. Williams *et al.* (1990), and Schäfer & Wöstemeyer (1992), also found that primers ending with either a G or a C at the 3' end were most efficient at promoting amplification in the species studied. They also found primers worked most efficiently if they were 9 or 10 bases long, and had a G+C content above 50 %.

Unfortunately, due to time constraints no further molecular biology could be performed on *C. perniciosa*. However, as a guide to future workers, any primers for testing should be either nonamers or decamers, have an A or a G at the 5' end, a G or a C at the 3' end, and the G+C content should be around 60-70 %.

7.1.1d Conclusions

The results conclusively proved that the RAPD PCR technique worked successfully in *C. perniciosa*. Of the 10 primers tested, one was completely successful, while 4 others worked in only one or two samples. Five primers did not promote any amplification.

Work must now concentrate on finding primers that allow the differentiation of *C. perniciosa* pathotypes. The search for a suitable primer should concentrate on nonamers and decamers, with an A or a G at the 5' end, a G or a C at the 3' end, and the G+C content should be around 60 %.

Fig. 7.1. Whole DNA extracts of primary and secondary phase *C. perniciosa* treated as described in the main text. Lanes labelled M contained molecular weight markers, size range 75-12216 bases. The DNA samples in lanes a, b, and c were extracted from the primary phase, those in lanes d, e, and f from the secondary phase. The plasmid band (P) is indicated.

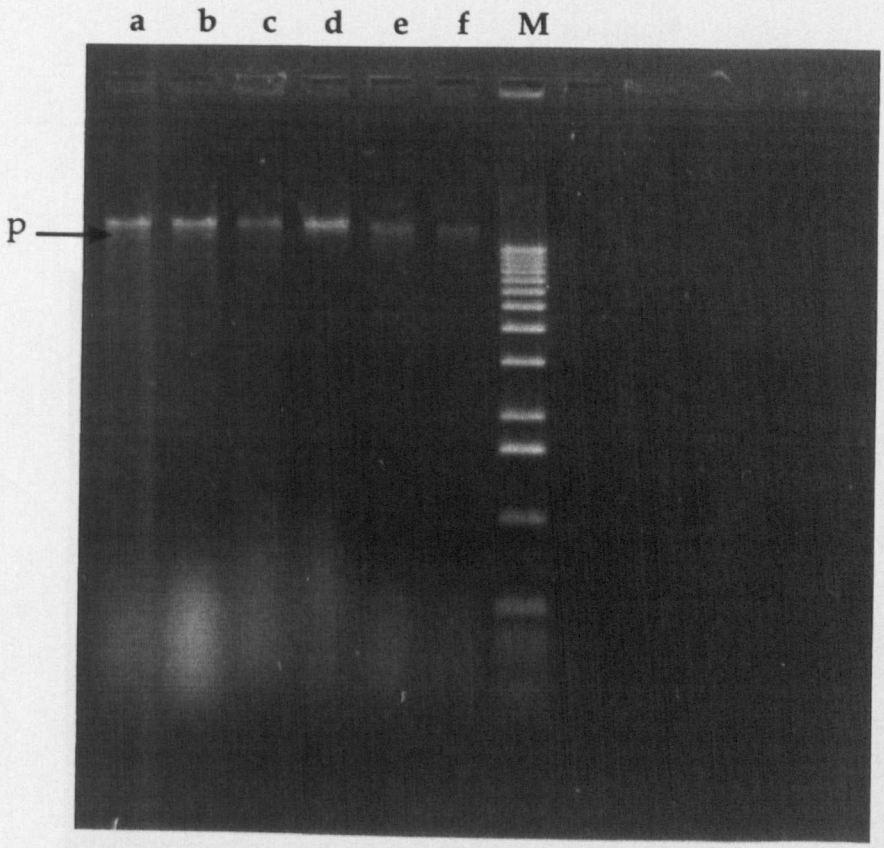


Fig. 7.2. DNA extracts from primary and secondary phase *C. perniciosa* amplified using the RAPD PCR technique described in the main text. Lanes labelled M contained molecular weight markers, size range 75-12216 bases. Lanes a and b contained PCR fragments generated from the primary phase using primer no° 3, with the sequence 5'-ACGGTCTTGG-3', lanes c and d contained PCR fragments generated from the secondary phase using the same primer. Lanes e and f contained PCR fragments generated from the primary phase using primer no° 4 with the sequence 5'-GCCGTAGTAC-3', lanes g and h contained PCR fragments generated from the secondary phase using the same primer. Lanes i and j contained PCR fragments generated from the primary phase using primer no° 5 with the sequence 5'-ACGATCGCGG-3', lanes k and l contained PCR fragments generated from the secondary phase using the same primer.

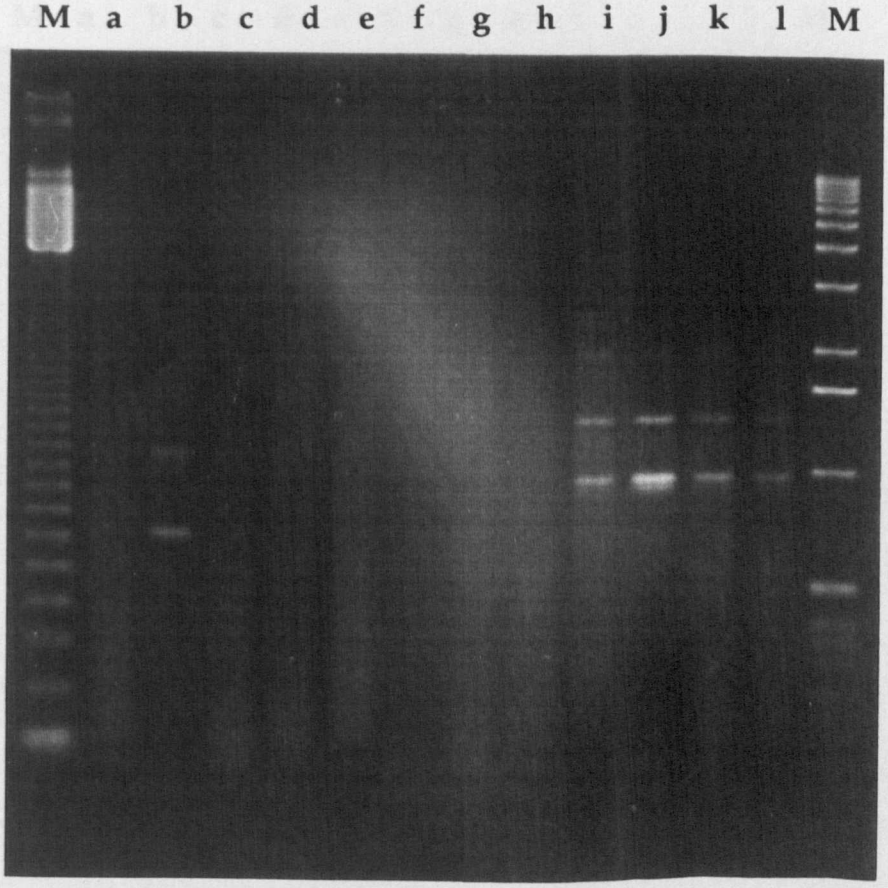


Fig. 7.3. DNA extracts from primary and secondary phase C. perniciosa amplified using the RAPD PCR technique described in the main text. Lanes labelled M contained molecular weight markers, size range 75-12216 bases. Lanes a and b contained PCR fragments generated from the primary phase using primer no° 6, with the sequence 5'-GAAACAGCGG-3', lanes c and d contained PCR fragments generated from the secondary phase using the same primer. Lanes e and f contained PCR fragments generated from the primary phase using primer no° 8 with the sequence 5'-GGAGCCCAC-3', lanes g and h contained PCR fragments generated from the secondary phase using the same primer. Lanes i and j contained PCR fragments generated from the primary phase using primer no° 11 with the sequence 5'-TGGGTCTAGT-3', lanes k and l contained PCR fragments generated from the secondary phase using the same primer.

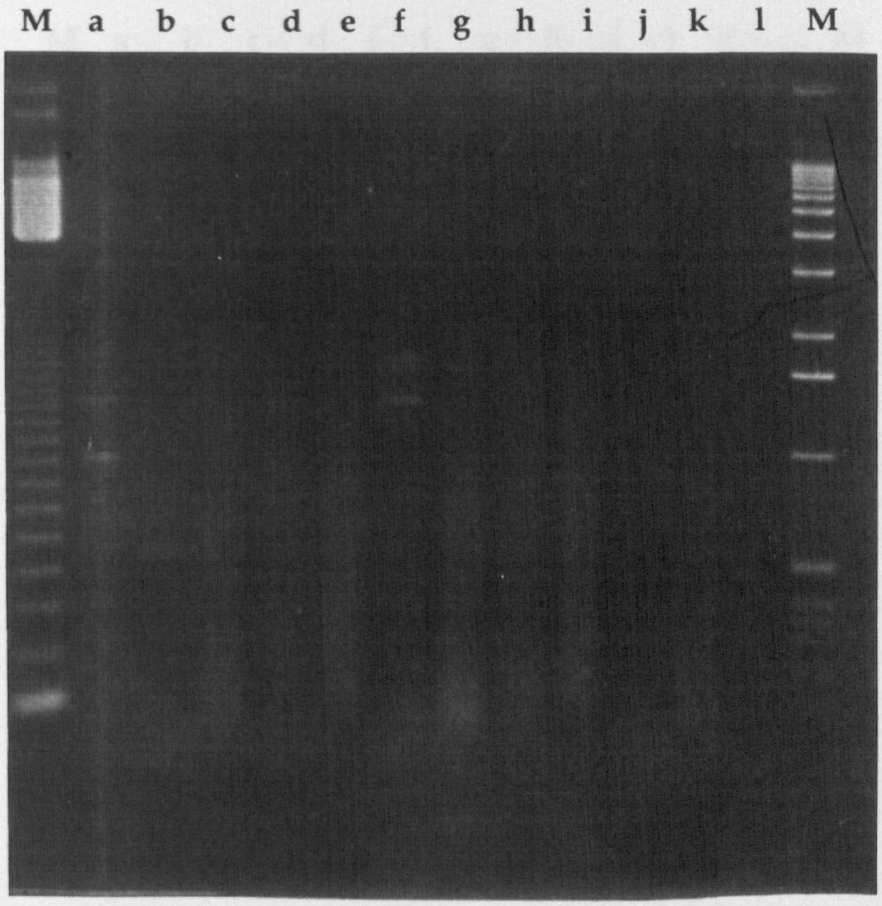


Fig. 7.4. DNA extracts from primary and secondary phase *C. perniciosa* amplified using the RAPD PCR technique described in the main text. Lanes labelled M contained molecular weight markers, size range 75-12216 bases. Lanes a and b contained PCR fragments generated from the primary phase using primer no° 14, with the sequence 5'-GCCGTCTACG-3', lanes c and d contained PCR fragments generated from the secondary phase using the same primer. Lanes e and f contained PCR fragments generated from the primary phase using primer no° 16 with the sequence 5'-CAATGGAGTG-3', lanes g and h contained PCR fragments generated from the secondary phase using the same primer. Lanes i and j contained PCR fragments generated from the primary phase using primer no° 17 with the sequence 5'-GGCATCGGCC-3', lanes k and l contained PCR fragments generated from the secondary phase using the same primer.

Chapter 8

Concluding remarks

The initial work in this thesis clearly demonstrated the ability to produce primary and secondary phase *C. perniciosa* in culture. This allowed biochemical tools for distinguishing between the primary and secondary phases to be developed and tested *in vitro*. A new and highly sensitive chitin assay was developed which allowed the study of *C. perniciosa* hyphae in sub-milligram portions of cocoa. The chitin assay could almost certainly be used in other plant fungal interactions. A new and improved mannan assay was also produced

All acid soluble sugars were evaluated as potential biomarkers for either the primary or secondary phases or simply for *C. perniciosa* as a whole. The sugars were also evaluated as part of potentially useful ratios which would allow the discrimination between the primary and secondary phases of *C. perniciosa* in infected cocoa tissue. Lipid components soluble in chloroform/methanol were likewise evaluated. Unfortunately, no useful ratios were discovered.

The new chitin assay was then used to study the distribution of *C. perniciosa* in green and brown brooms. Brown brooms were shown to contain greater *C. perniciosa* biomass than green brooms. More importantly, *C. perniciosa* was shown to be concentrated in the bud tissue and at the base of a broom. The distribution of *C. perniciosa* in a broom may indicate that the fungus induces an environment suited to each phase of its life cycle. The primary phase requires an expanding food source and is associated with the rapid production of additional cocoa tissue. The secondary phase requires dead cocoa tissue and is associated with green broom death and the production of brown brooms. The brown brooms subsequently dry out. The secondary phase is better adapted to the drier environment than its competitors. Thus, all the circumstantial evidence indicates *C. perniciosa* creates an environment uniquely suited to itself, at the expense of cocoa and other potential pathogens or saprotrophs.

Future work should concentrate on using the new chitin assay to study the distribution of *C. perniciosa* in infected cocoa material. The chitin assay proffers a unique opportunity to study the growth patterns of *C. perniciosa* in infected cocoa. Its unique feature is the achievable accuracy and sensitivity which allows the detailed distribution of fungus to be determined

within extremely well defined areas of the host. At present, no other method is capable of achieving this aim in such a sensitive and accurate manner.

The groundwork has also been laid for the study of *C. perniciosa* using the latest advances in molecular biology. The method, outlined in Chapter 7 and carried out at IGF Berlin, will allow the distinction between different *C. perniciosa* pathotypes. If the successful primer detailed in Chapter 7 does not allow the discrimination between different pathotypes then other random primers almost certainly will. Work should also concentrate on using this method as an integral part of the planned cocoa resistance breeding programme. An evaluative tool capable of quickly discriminating between different *C. perniciosa* pathotypes would prove to be invaluable as part of such a programme.

One intriguing feature highlighted by the molecular biology work was the presence of fungal plasmids in the primary phase of the fungus, but not in the secondary. Plasmids are extremely rare in fungi. It would also be interesting to determine whether plasmids play any role in pathogenicity.

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