Disease resistance in cocoa germplasm: Witches' broom disease

Keith Hardwick, Danny Penman, Hamish Collin and Susan Isaac

School of Life Sciences, University of Liverpool,
P.O. Box 147, Liverpool, L69 3BX, U.K.

Successful cultivation of any commercial crop must achieve both acceptable quantities and qualities of harvested product. Disease interferes with potential harvest achievements. Identification or production of germplasm resistant to target disease(s) is therefore part of any germplasm development programme. Such programmes are built upon basic understanding of the epidemiology and physiology of the interactions between host and disease-causing organism. One research programme at Liverpool University aims to extend the very limited knowledge of the interaction between the fungus causing witches' broom disease, *Crinipellis perniciosa* (Stahel) Singer, and the cocoa host. With improved knowledge, development of resistant germplasm, and more effective disease control measures should soon follow. At present we do not know how the fungus develops within the host or how variable disease symptoms, from mild to severe, relate to fungal biomass and activity within the host. An added complication is the two-phase life cycle of the fungus: a primary, monokaryotic, biotrophic phase followed by a secondary, dikaryotic, saprotrophic phase. Progress and disease severity are likely to be related to different amounts of the two phases and the timing of dikaryotization. This research aimed to develop chemical analyses to quantify each hyphal growth phase during the progress of the disease and in different levels of disease severity. The analyses evaluated were based on fungal-specific mannan, chitin, sterols and lipids. Mannan concentrations and mannan:chitin ratios differed significantly between phases, but surprisingly, uninfected host tissue had high mannan levels, and thus mannan concentration cannot be the sole, or a component of, a measure of fungal biomass. Previous chitin analyses were too insensitive. A new GC based method with some x 1500 increased sensitivity has been developed. The new method is capable of measuring nanogram levels of fungus in sub-milligram portions of cocoa. It should now be possible to achieve accurate quantification in a range of infected material. The fungal sterol, ergosterol, often used to measure fungal biomass in diseased plants, is not present in healthy cocoa tissue. It is easily quantified by GC, and evaluation of its potential for fungal quantification in diseased cocoa tissues is under way. The possibility of using other sterols and lipids, including ones already found to differ between hyphal phases, is also being evaluated.

Introduction

The tropical cocoa plantation environment is subject to significant and diverse pressures from diseases. One fungal pathogen of particular significance is *Crinipellis perniciosa* (Stahel) Singer, which causes witches' broom disease of cocoa. This disease occurs throughout South America and the Caribbean where it can cause crop losses approaching 100% in bad years. Thankfully the disease has so far failed to spread outside the Americas, largely because of stringent quarantine procedures (Wheeler, 1985). Attempts have been made to identify

resistant cultivars of cocoa (Fonseca and Wheeler, 1990), but little natural resistance has been found, and this has all too frequently been lost in cocoa plantations. The need for more resistant cultivars is great, especially as witches' broom is now spreading rapidly throughout Bahia, an extremely important cocoa producing region of Brazil.

The unusual life cycle of *C. perniciosa* has been described by Evans (1980). Basidiospores germinate to produce primary monokaryotic mycelia which penetrate the host, growing intercellularly and living biotrophically. Primary phase mycelium is associated with the distorted growth of green brooms. After some months the fungus dikaryotises to produce the secondary phase which now grows intercellularly and saprotrophically. The fungal life cycle is completed after 6-9 months in the field (Rudgard, 1986) when basidiospores are released from basidiocarps growing on the dead, brown broom material.

One of the main deficiencies in our knowledge of the cocoa: *Crinipellis* interaction is the amount of fungal biomass that occurs within the host. For example, disease symptoms vary enormously, but it is not known how these relate to fungal biomass or to the relative proportions of primary and secondary phase mycelium within the host. We also have no information concerning the timing of dikaryotisation and the effect of this change on disease symptom development.

Estimation of fungal biomass within higher plant hosts has been undertaken in the past using fungal specific compounds such as chitin, mannan and lipids as markers. Estimation of fungal biomass using chitin is well documented (Golbuchuk *et al.*, 1960; Ride and Drysdale, 1971, 1972; Swift, 1973; Lung-Chi Wu and Stahman, 1975; Donald, 1977; Nandi, 1978; Chen and Johnson, 1983; Whipps, 1987). However the validity of a chitin assay alone has been questioned by Sharma (1977). Whipps *et al.* (1980) overcame Sharma's criticism by using chitin assays for a comparative measure rather than an absolute measure of fungal biomass. Thus Whipps *et al.* (1982) assessing the growth and development of *Puccinia hordei* (Otth.) on barley, found that chitin and also mannan concentrations, correlated with histological observations of fungal development, and both compounds proved to be good indicators of fungal growth and development. Later studies by Whipps *et al.* (1985), showed that the mannan to chitin ratio shifted prior to sporulation of *Puccinia* which indicated that a major developmental change during the life cycle was accompanied by a significant biochemical change. The original method for chitin estimation developed by Tsuji (1969) was based on a colorimetric procedure and adapted for use in plants by Ride and Drysdale (1972). There are numerous disadvantages associated with this colorimetric procedure, the most significant being lack of sensitivity and specificity. The use of gas chromatography, combined with a mass spectrometer, would overcome these problems.

An alternative approach to assessing amount and change of the fungal biomass in host tissue is to use lipid, and more specifically, sterol analysis. Sietz *et al.* (1979) found that an ergosterol assay for measuring fungal invasion in stored cereal grains was more sensitive and reliable than the existing chitin assay. Lumsden *et al.* (1990) noted that the ergosterol content of the pathogen *Trichoderma harzianum* Rifai varied with culture age and increased at sporulation. Analysis of Eucalyptus roots infected by *P. tinctorius* (Alb. and Schwein) showed that ergosterol was useful for detecting and monitoring the invasion process. Where the relative amounts of key cellular components vary with fungal development, the change in ratios of such compounds can be used to identify the change more clearly. It was important therefore to determine the lipid profile of *C. perniciosa* in both the primary and secondary phase. This would allow identification of any lipids specific to each growth phase, and highlight any lipid that might form part of a ratio to identify the separate phases in the absence of any qualitative difference in lipid composition.

The aim of the research described here was to examine and improve biochemical methods for quantifying fungal material in cocoa tissues infected with *C. perniciosa*. Ideally such techniques would then allow the separate quantitative assessment of both the primary and secondary phases of the fungus within the host. The investigations concentrated on the fungal marker compounds, chitin, mannan and lipids. Axenic cultures of the primary phase and secondary phase mycelium separately, were grown to provide fungal material for an initial examination of chitin, mannan and lipid levels to establish the presence of differences in composition between the phases of the fungus in the absence of host tissue. Subsequently the investigation was extended to broom tissue.

Methods

Only outline details of methods are presented here.

1. Production of fungal and plant material

Cultures of the primary phase were initiated by suspending mature basiodiocarps from the lids of glass universal bottles and allowing spores to drop into potato dextrose liquid medium for germination. The primary phase mycelium lacking clamp connections was maintained for up to 35 days at 20°C with a 16 hr photoperiod. Fragmented secondary phase mycelium grown on V-8 juice agar was used as inoculum for liquid cultures. Fragments were transferred to liquid potato dextrose medium and grown in shake culture under the same conditions of temperature and light. Cultures of *C. perniciosa* were initiated with Manaus F, an isolate kindly provided by Dr. B.E.J. Wheeler, University of London.

Greenhouse-grown two year-old West African Amelonado seedlings were used to provide disease free cocoa shoot apices. Diseased material was also imported from Bahia, Brazil and Trinidad as brown and green brooms, under license.

2. Chitin and mannan assay

The estimation of the polymers, chitin and mannan, was based on analysis of the acid hydrolysis products glucosamine and mannose respectively. Calculations of amounts of chitin and mannan were therefore based on amounts of the respective monomers.

Chitin and mannan were hydrolysed from the cell walls of fungus, healthy cocoa and broom tissue using 6M hydrochloric acid and 2M trifluoroacetic acid respectively. The hydrolysis products including glycosamine or mannose were reduced and acetylated, then cleaned by partitioning between a series of polar and non-polar solvents. Separation and quantification of glucosamine and mannose from the other components were achieved with a Hewlett Packard 5890 Series 2 Gas Chromatograph fitted with a BP 10 capillary column. Identification of components was confirmed using a VG Mass Spectrometer. Subsequently ratios of mannan:chitin were calculated.

3. Lipid and sterol assay

Lipids were extracted from both fungal and plant material using chloroform and methanol. Plant samples were cleaned using thin layer chromatography before derivatization; fungal samples did not require cleaning. The lipids were derivatized using BSTFA immediately prior to gas chromatography. The separation of the lipids was achieved with the same Gas Chromatograph, but fitted with a B5 column. Identification of the compounds was achieved by mass spectrometry.

The quantitative data was analysed statistically by noting all compounds which occurred at a concentration of 250 $ng.ml^{-1}$ dry tissue and carrying out a one-way analysis of variance on these compounds to identify those which differed quantitatively between the fungal phases. Ratios between all combinations of compounds which differed significantly were calculated and again assessed for significance with a one-way analysis of variance. Subsequently healthy cocoa tissue was screened to see whether it contained compounds which would interfere with the application of a ratio analysis in diseased tissue.

Results and Discussion

1. Mannan:chitin ratio in fungal and plant tissue

A significant criticism of the colorimetric procedure for chitin analysis is that it was not sufficiently sensitive or specific. Using the gas chromatographic method, the detection limit was about 50 pg of glucosamine which was 1500-1700 times more sensitive than the colorimetric procedure. The gas chromatograph gives a clear separation of glucosamine (Fig. 1a) which means that the quantitative data represent a direct measurement of glucosamine, rather than the measurement being affected by a range of six-carbon amino sugars in the colorimetric method. In addition, the new assay requires only very small amounts of tissue so that highly specific areas of a given biological material can be analyzed. The mannan estimation used here (Fig. 1b) is probably not significantly more sensitive than the method developed by Jones and Albershein (1972) and used by Whipps *et al.* (1980, 1982, 1985), but combination of the methods for preparation and separation of glucosamine and mannose means that these assays are more directly comparable.

The ratios of mannan to chitin in the primary and secondary growth phases are given in Table 1. Interestingly there is a wide difference between the mannan:chitin ratio in the primary phase (4.53) and in the secondary phase (2.60). The concentration of mannan in the secondary phase was less than in the primary phase, whereas the concentration of chitin was very similar in both growth phases, at 14.83 ± 0.77 $\mu g\ mg^{-1}$ for the primary and 17.02 ± 0.63 $\mu g\ mg^{-1}$ for the secondary phase. Since there was a relatively small difference in chitin concentrations between the primary and secondary phases of *C. perniciosa*, chitin content may be used as a measure of invasion into cocoa tissue. This conclusion concurs with that of those working on other fungi (Golubchuk *et al.*, 1960; Ride and Drysdale, 1971, 1972; Swift, 1972; Lung-chi Wu and Stahman, 1975; Donald, 1977; Nandi, 1978; Chen and Johnson, 1983; Whipps, 1987).

Figure 1a Gas chromatograph of the mannan extraction from the secondary phase of *C. perniciosa*. Note position of mannose (M)

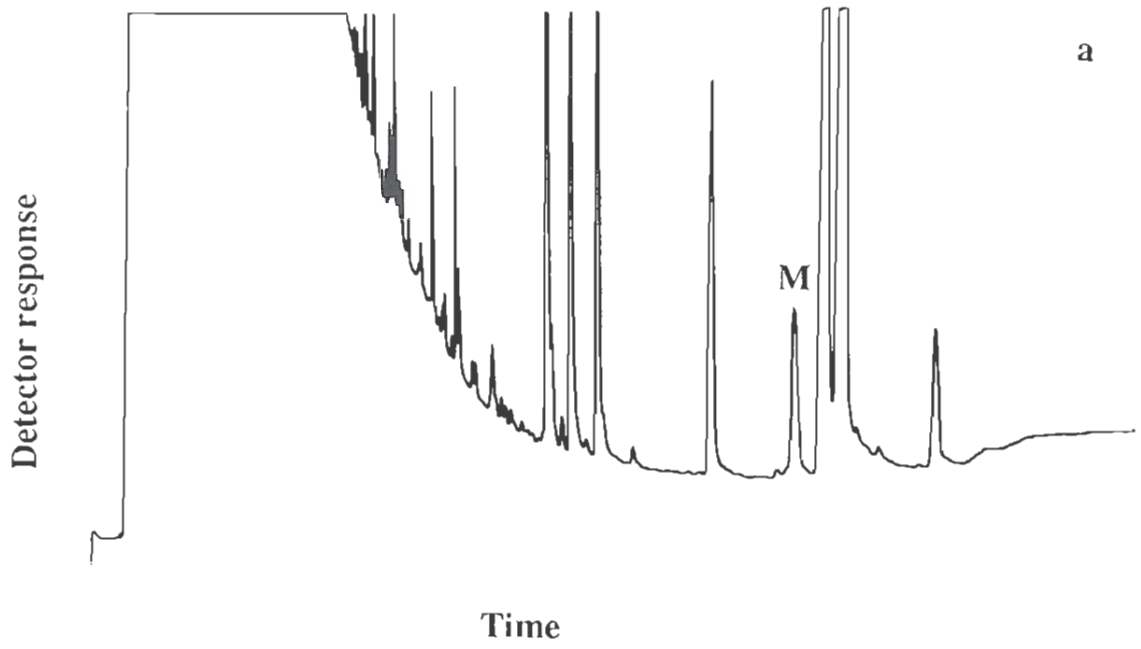


Figure 1b Gas chromatograph of the glucosamine extraction from the secondary phase of *C. perniciosa*. Note position of glucosamine (G).

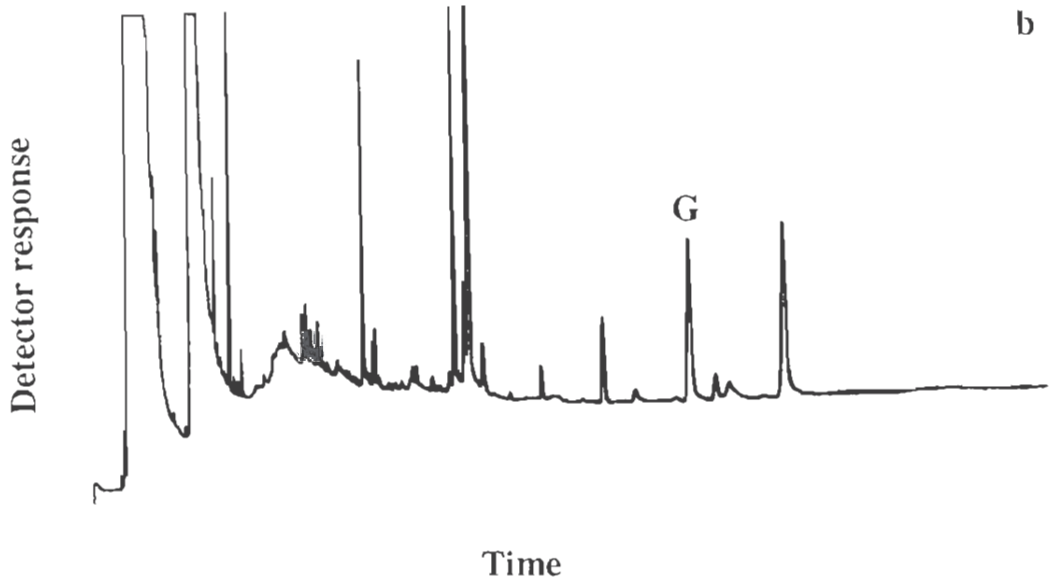


Table 1 Chitin and mannan content and ratio in cultures of *C. perniciosa* and healthy and diseased cocoa tissue.

Source Material	Chitin or mannan content (ug.mg^{-1} tissue ± S.D.)	Mannan: Chitin
Primary stage *C. perniciosa*	Mannan 67.12 ± 6.15 Chitin 14.83 ± 0.77	4.53
Secondary stage *C. perniciosa*	Mannan 44.29 ± 4.85 Chitin 17.02 ± 0.63	2.60
Healthy stem apex *Theobroma cacao*	Mannan 7.77 ± 1.26 Chitin 0.03 ± 0.01	247.57
Green broom	Mannan 6.58 ± 0.46 Chitin 1.27 ± 0.18	5.18
Brown broom	Mannan 8.66 ± 0.25 Chitin 2.32 ± 0.23	3.73

Before attempting to estimate fungal levels in diseased cocoa tissue it was necessary to screen uninfected cocoa tissue for glucosamine and mannose. Presence of these compounds in host tissues will not necessarily invalidate the approach to quantifying fungal levels, but any significant 'background' levels of these compounds would have to be taken into account in setting practical detection limits for the fungus. The results in Table 1 show that chitin constituted about 15 ug mg^{-1} dry weight of fungus and only about 30 ng mg^{-1} (measured as glucosamine) dry weight of healthy cocoa tissue. The background level of glucosamine in cocoa is probably due to its liberation from glycoproteins during the acid hydrolysis stage of the extraction procedure (Fox, pers. comm.). This means that a realistic detection limit for *C. perniciosa* (in cocoa tissue) is equivalent to approximately 40 ng mg^{-1} chitin per mg cocoa which is equivalent to about 0.3% w/w of fungal tissue within a diseased cocoa sample, quite a low level of fungus. Rocha (1985) using the Tsuji (1969) based colorimetric procedure estimated the chitin concentration in *C. perniciosa* to be about 8.5 ug ml^{-1} dry weight, a figure somewhat lower than that obtained here.

The data (Table 1) indicate that there was a very high background level of mannan in cocoa tissue constituting about 0.7% dry weight. Mannans are known to occur in hemicellulose components of higher plant cell walls, but other workers using mannose assays for other species have not found high levels (Whipps *et al.*, 1985). Mannans, especially galactomannans, are also known to be major components of many plant gums and mucilages (Aspinall, 1969). Cocoa is known to be a prolific producer of mucilage and it is possible that the high mannose levels in cocoa may derive from this. Due to the high background concentrations of mannan in cocoa, a mannan:chitin ratio may be used to indicate the presence of primary and secondary phase *C. perniciosa* where total fungal loading constitutes more than about 9% of the total dry weight of a diseased tissue sample. A chitin assay however may be used if the concentration of fungus is likely to exceed 0.3% w/w of diseased cocoa tissue.

Figure 2 Gas chromatograph of the glucosamine from the primary phase of *C. perniciosa*. Peaks represent lipids, hexose sugars and sugar alchols

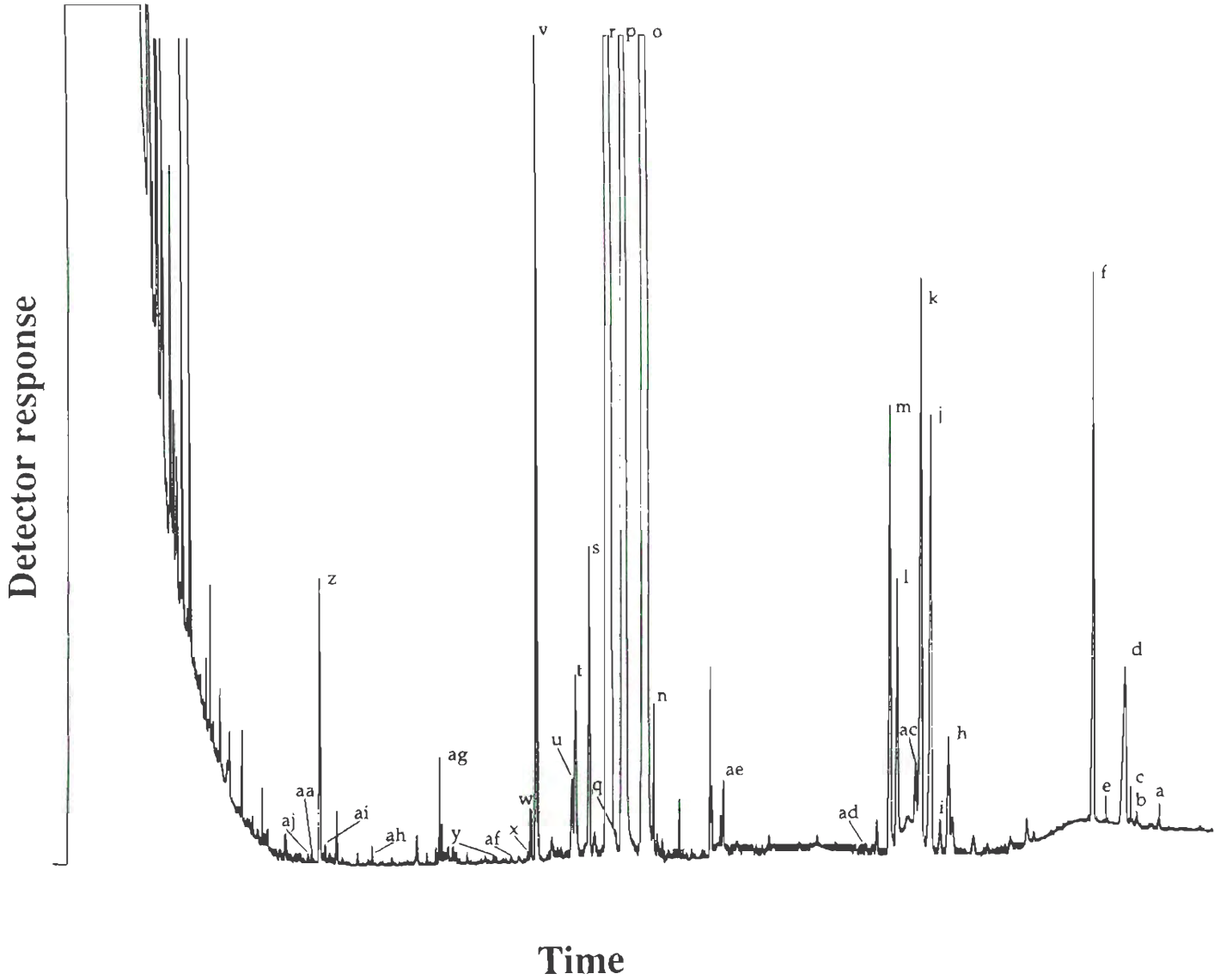
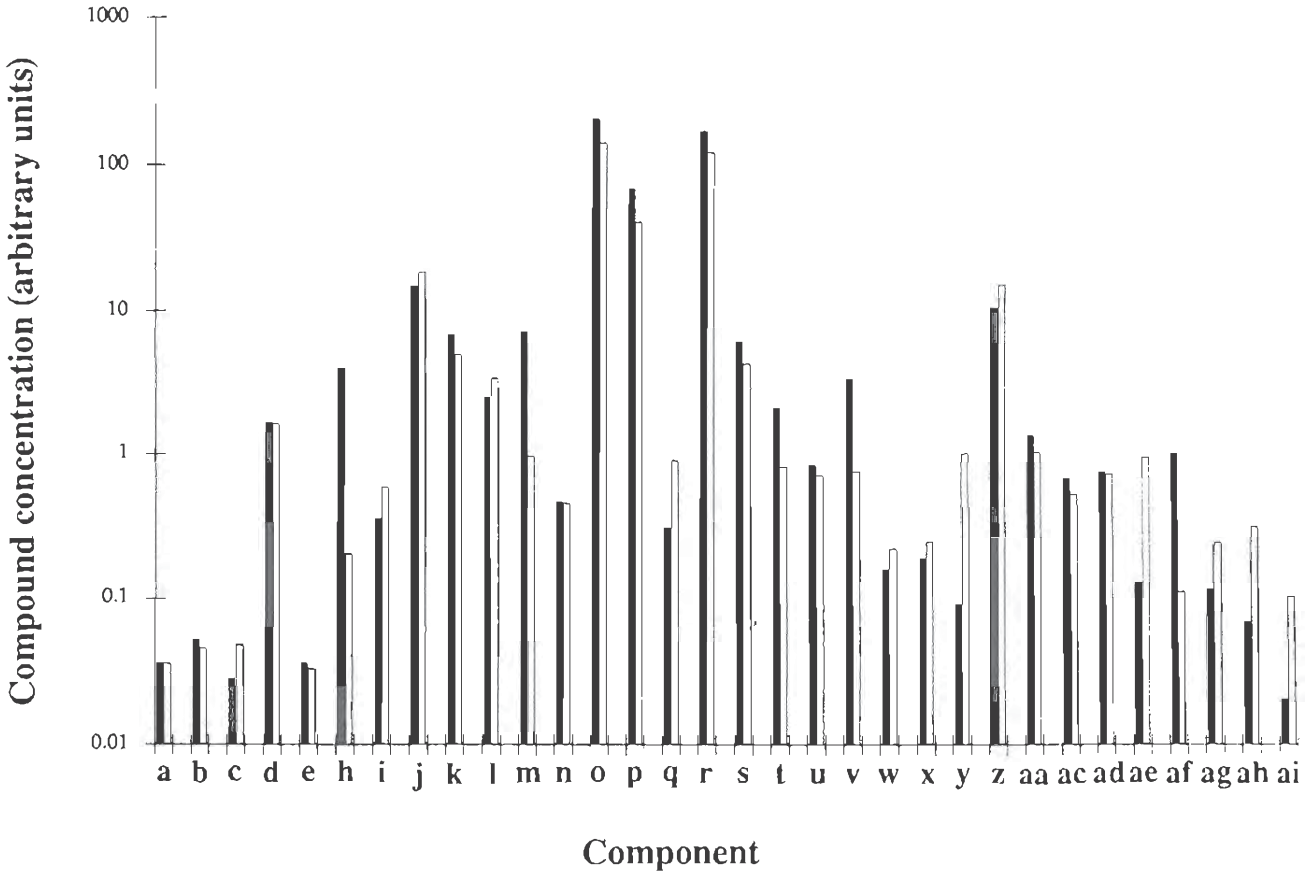


Figure 3 The relationship between the concentrations of compounds in the lipid extraction from both the primary (solid bars) and secondary (open bars) and phases of *C. perniciosa.*



In spite of the limitations of the mannan and chitin analysis for investigation of diseased cocoa tissue, when green and brown brooms were analysed some very interesting features emerged. Results from Table 1 show that the average mannan:chitin ratio is higher for green brooms (5.18) than from brown brooms (3.73). This pattern is what would be expected from the mannan:chitin ratios determined for the two phases of *C. perniciosa* from axenic cultures, and the assumption that the primary phase is thought to predominate in green brooms and the secondary phase in brown brooms.

2. Lipid profile

A representative lipid profile for the primary phase and secondary phase of *C. perniciosa* is shown in Figure 2. Thirty-three peaks were identified as representing compounds with concentrations greater than 250 ng ml^{-1} of fungal mycelium (a-ai).

Statistical analysis showed that components 'h' and 'ae' formed a potentially useful ratio pair. The h:ae ratio in the primary phase was 32.5:1, whilst in the secondary it was 0.04:1.

Component 'h' was identified as squalene, but component 'ae' has not yet been positively identified. No lipid ratio pairs were identified which could be used to distinguish between the primary and secondary phases.

Ergosterol (compound d) was found to be a major lipid component of both primary and secondary phases of *C. perniciosa* with a mean concentration of 1.66 ug mg^{-1}. It is completely absent from healthy cocoa tissues. Ergosterol is thus potentially a very sensitive means of detecting *C. perniciosa* in cocoa tissue. Where cocoa or other plant materials only need to be screened for the presence or absence of fungal material, the ergosterol assay would be a highly sensitive and specific approach.

Conclusions

1. A highly sensitive and specific method was developed for chitin estimation. This method permits measurement of fungal tissue within milligram quantities of host tissue.

2. There was a major difference in the mannan:chitin ratio between the primary and secondary phases of *C. perniciosa.* This derives from a significantly lower concentration of mannan in the secondary phase.

3. It is now possible to use the mannan:chitin ratio to estimate fungal biomass in diseased tissue where the fungal biomass constitutes more than 9% w/w dry weight. The chitin assay however, can be used to assess fungal biomass as low as 0.3% w/w dry weight of fungus in host tissue.

4. Comparison of the mannan:chitin ratio in green and brown brooms identified the predominant phase of development of *C. perniciosa* in those tissues.

5. It was not possible to distinguish the primary and secondary phases on the basis of lipid compounds.

6. Ergosterol was a major lipid component of both phases of *C. perniciosa* and was totally absent from healthy cocoa tissue. This assay could form a rapid and sensitive method for measuring fungal biomass in diseased tissue.

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