STUDIES ON THE DEGRADATION OF TESTA OF COCOA (*THEOBROMA CACAO* L.) BY *ASPERGILLUS* SPECIES ISOLATED FROM MOULDY COCOA BEANS

A Thesis presented by

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In partial fulfilment of the requirements

for the M.Sc. Degree of the

University of Ghana

JULY, 1982

From: The Department of Botany

University of Ghana

LEGON.

I hereby declare that the work presented in this thesis:

STUDIES ON THE DEGRADATION OF TESTA OF COCOA
(THEOBROMA CACAO L.) BY ASPERGILLUS SPECIES
ISOLATED FROM MOULDY COCOA BEANS"

was done entirely by me in the Botany Department, University of Ghana, Legon.

This work has never been presented either in part or completely, for any degree of this University or elsewhere.

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ABSTRACT

Cocoa beans at the Tema Warehouses were found to be contaminated with a total number of 21 fungus species, the predominant species belonging to the genus Aspergillus. The annual average contamination was 4.9 per cent. Infection in the main crop was lower (3.9 per cent) than in the minor crop (6.4 per cent). The higher the bean moisture content the greater the fungal infection.

Extracts of testa of fermenting cocoa beans depressed vegetative growth in Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus tamarii and Sclerotium rolfsii. S. rolfsii was more inhibited than the Aspergillus species. Extracts of testa of beans already fermented and being dried had no effect on growth of mycelium of the Aspergillus species but stimulated that of S. rolfsii. A. fumigatus grew best in Sabouraud's broth medium while A. tamarii grew best in Czapek Dox broth medium.

Cotyledons and testa of cocoa beans possessed phenolic compounds; free phenols, orthodihydric phenols, flavonols and anthocyanins. The quantities of these compounds changed in different ways during curing of the beans. Free phenols content in both cotyledons and testa decreased during fermentation and drying of the beans. Orthodihydric phenol levels in both cotyledons and testa, on the other hand, increased during fermentation and decreased during drying of the beans. Flavonol content in the cotyledons increased during fermentation and decreased during drying, while it decreased in the testa during both fermentation and drying. The anthocyanin content of the cotyledons decreased with curing but increased significantly in the testa.

Some of the phenolic compounds inhibited germination of conidia and germ tube growth in A. flavus, A. fumigatus, and A. tamarii and germination of sclerotia of S. rolfsii to varying degrees. Others, however, stimulated germination. The compounds in either case were not identified.

Fungal contamination of cocoa beans was observed in unfermented fresh cocoa beans and in beans at various stages of fermentation and drying. Invading fungi occupied mainly the micropylar and stylar regions of the beans. The composition of the flora changed as fermentation and drying of the beans progressed.

CONTENTS

I. INTRODUCTION

Cocoa produced in Ghana, is accepted all over the world as being of very high quality. Writing on the cocoa bean characteristics that make for good quality cocoa, Wood (1979) stated .... "it is unusual for any cocoa to exceed the normal figures for Ghana cocoa, so, on this basis, as on others, Ghana is the premium on cocoa". He listed the unacceptable defects of merchantable cocoa beans as mouldiness, slatiness, level of insecticidal chemical residues, insect damage, foreign odours (smoke) and foreign materials (stones, pieces of wood, etc). It is recommended that commercial cocoa beans be dried to a moisture level below 8 per cent so as to reduce mycofloral development (Dade, 1929). Of these defects, mouldiness is the most objectionable and has been described as by far the worst defect of commercial cocoa (Wood, 1965).

Guidelines set out by the Working Party on Cocoa Grading of the Codex Alimentarius Commission, give acceptable standards that commercial cocoa has to meet (Anon, 1969). In Ghana, relevant regulations delimit three grades of cocoa beans for export. Grade I cocoa beans should not have more than 3 per cent internal mouldiness, and beans with 4 - 8 per cent mouldiness are classed as Grade II (Anon, 1968). Exportable cocoa beans with more than 8 per cent internal mouldiness is graded as sub-standard and sold as inferior cocoa. Thorold (1975) put it most succinctly when he stated that "the objective of increased productivity is not satisfactorily achieved unless the produce is marketable."

Mouldiness in stored food products is known to pose serious public health problems. Some of the commonly occurring fungi are known to produce mycotoxins (Mayne, Bennett, Tallant, 1971). One notable example is the death of 100,000 turkey poults in England in 1960 which was traced back to groundnuts infected with Aspergillus flavus and fed to the poults (Spensley, 1963). Although Wood (1979) has assured the chocolate consuming public that none of the fungi found on cocoa beans has been found to produce mycotoxins in the beans, the presence of the fungi has other unfavourable consequences.

Of great concern to chocolate manufacturers is the flavour of cocoa beans after roasting. The flavour is markedly affected by mouldiness. In the event of heavy fungal contamination, the beans become "off" flavour which can only be partially corrected through expensive deodorising processes. In common with stored seeds of other plant species (Milner and Geddes, 1946; Altschul, 1948) infection of cocoa beans by fungi causes an increase in the free fatty acid content of cocoa beans (Maclean, 1953). This is undesirable since it reduces the amount of valuable fat of the beans.

In order to minimise the incidence of moulds on the beans in storage, it is recommended that cocoa beans be dried to 8 per cent moisture content. The cocoa bean is hygroscopic and absorbs or looses moisture to get into equilibrium with ambient relative humidity. In sorption isotherms, Scott (1931) showed the relationship between relative humidity and moisture content of cocoa beans. At 26°C and 84% R.H., the equilibrium moisture level in cocoa is 8.4 per cent and at 82% R.H., the moisture level is 8.0 per cent. On the basis of these derivations, Dade (1929) and later Knapp (1937) recommended that at 26°C, the relative humidity conditions for storage of prepared cocoa beans should be at most 82% R.H. Aspergillus glaucus, one of the fungus species most frequently found in mouldy beans and most tolerant of low moisture levels cannot survive below these conditions of temperature and relative humidity. The recommended 8 per cent moisture content is regarded as an essential lower limit, for, below this threshold, the beans become brittle and are easily broken and invaded by insect pests.

Bunting (1929) studied the mycoflora of cocoa beans in Ghana. The most important species he found belonged to the genera Aspergillus, Rhizopus and Mucor. Some of the storage fungi invade the heap of fermenting cocoa beans, encouraged by the sugary mucilage of the beans. Many of the initial invaders will be eventually killed by the high temperature (about 50°C) of the fermentation process (Rohan, 1963; Oyeniran and Adeniji, 1974), but the thermophilic species, such as Aspergillus fumigatus and Mucor buntingii survive. The development of these thermophilic species is encouraged by prolonged fermentation beyond the six-day period which is normally used (Rohan, op. cit). Poorly fermented beans often begin to germinate and the crack caused in the testa by the emerging radicle creates an entrance for fungi growing on the surface of the bean. Rohan (1963) has suggested that the four-day method of fermentation could be used, with adequate mixing of the beans to produce cocoa beans with acceptable flavour when roasted. The observation by Dade (1929) that unfermented beans are mostly free from internal moulds would support the proposition that internal mould development mainly starts during fermentation. Infection of beans in the pod is, however, possible. Ekundayo (1970), for example, observed that beans in the pods were infected by *Botryodiplodia theobromae*. Many fungi invade the cocoa beans during drying after fermentation. The usual humid conditions (85 - 100% R.H.) of the cocoa farm where drying is carried out could prolong the drying period to 12 - 14 days and encourage fungal contamination. There is a lower occurrence in the incidence of internal mouldiness of the main-crop cocoa harvested during the drier (Harmattan) months of January to March when drying is more rapid than the light-crop cocoa harvested during the humid months of May/June/July when drying takes a longer time (Dade, 1927). Scott (1931) observed that a drying period of 10 - 12 days caused by rains and cloudy conditions increased fungal infection of the cocoa beans by 30%. In Malaysia, quick artificial drying has been suggested as the best approach to reduce contamination (Anselmi, Liu and Mu, 1974). If artificial drying is to be recommended so as to cut down the time taken to dry fermented beans, the cocoa will require a "maturation" period of 2 days between the fermentation and drying processes to fully develop the desired chocolate flavour precursors (Liau, 1976).

Analyses of the cocoa bean has shown that it has a varied tannin/polyphenol content (Forsyth, 1952b, 1954; Griffith, 1958; Swain, 1960). It has been shown that quantitative and qualitative changes in the chemical constituents of the bean take place during fermentation. These changes lead to a varied array of hydrolysis and polymerisation products of the phenolic component (Huang, 1955; Roelofsen, 1958; Rohan, 1963). Some chemical constituents of plants such as alkaloids, volatile acids, indole acetic acid and polyphenols have been suggested as being preformed toxicants against infection agents. Polyphenols, however, have been directly implicated in plant disease resistance mechanisms (Pridham, 1960; Kuc, 1964; Kosuge, 1969). Since unfermented cocoa beans are immune to internal moulds, it is of interest to find out if the tannins and polyphenols in cocoa play any role in disease prevention.

This thesis contains work carried out on the chemical changes that occur in the phenolic component of testa during cocoa curing. The effects of individual phenolic compounds on the growth characteristics of conidia of <u>Aspergillus</u> species isolated from mouldy beans was investigated. The results of this study are necessary because they would show specific preformed chemical inhibitors, which occur in cocoa bean testa, and the effect of curing on these compounds.

II. LITERATURE REVIEW

The cocoa bean consists of two cotyledons forming about 87 per cent of the seed. These cotyledons are enclosed in the testa or seed coat which forms twelve per cent of the dry weight of the seed while the germ or embryo forms one per cent of the seed (Knapp and Coward, 1934). The seed coat or testa, sometimes called the skin, shell or husk is made up of two integuments fused together. The outer one contains some vascular bundles and longitudinal rows of big cells with very thick mucilagenous cell walls.

An anatomical examination of the cotyledon by a process of differential centrifugation (Brown, 1954), showed that it is made up of two types of cells, Large polyphenol storage cells constituting 10 - 13% of the tissue, usually in groups, and scattered among parenchymatous cells which contain enzymes, proteins and lipid droplets. They contain the purine bases and have high amounts of anthocyanins.

The oxidation of these phenolic compounds by an enzyme complex leads to the formation of products which impart flavour to manufactured chocolate and chocolate products (Wood, 1975). This oxidation forms part of the specific processes of curing (ie fermentation and drying) and roasting which produce the characteristic chocolate aroma (Swain, 1960). Curing of the cocoa bean is initiated by microbial fermentation by yeasts followed by lactic acid and acetic acid bacteria (Roelofsen, 1958). During the process of fermentation, the bean is killed by the combined action of acetic acid and ethanol and the high temperature of 51°C within the fermentation mass. (Roelofsen and Geisberger, 1947; Oyeniran and Adeniji, 1974). Consequently, the cells lose their semi-permeability, and phenolic compounds diffuse into the parenchymatous cells where enzymatic oxidation initiated by polyphenol oxidase begins. This oxidation continues at a slow rate due to the largely anaerobic conditions created by the breakdown of pulp tissue and the exclusion of oxygen from the mass. As the sweatings drain out of the fermentation heap, and also during drying, prevailing aerobic conditions accelerate the oxidation process (Forsyth and Quesnel, 1957).

At the beginning of fermentation, the mass of seeds is composed of pulp and beans. Pulp is made up of 80 - 90% water and 8 - 13% sugars made up of glucose (8 - 10%) sucrose (0.4 - 1%) as well as raffinose and stachyose (Thaler, 1954, Forsyth and Quesnel, 1963). Sucrose levels reduce during fermentation due to hydrolysis and dissimilation as well as migration into the testa (Howat, 1957). Howat (op.cit) reported that cocoa beans contain 0.2 to 0.4% (w/v) of non-volatile acids like citric acid, oxalic acid, acetic acid and that these contribute to the acidity of the pulp (pH 3.6 - 3.7) tissue. In contrast to the pulp, the bean is composed of water (33%), cocoa butter or fat (33%), and starch, sugars, purine bases (theobromine and caffeine), enzymes, amino acids and phenolic compounds (33%) (Forsyth and Rombouts, 1951; Roelofsen, 1958; Rohan, 1963). The phenolic compounds fall under three main groups; catechins (37%), anthocyanins (4%) and the leucoanthocyanins (58%). The main catechins are epicatechins, gallocatechins, and epigallocatechins. The anthocyanins are 3-α-L arabinosidyl cyanidins and 3-α-D galactosidyl cyanidins (Forsyth, 1952b; Griffith, 1958; Roelofsen, 1958; Rohan, 1963). Leucoanthocyanins in cocoa occur as the aglucon leucoanthocyanin and so do not contain sugar residues (Robinson & Robinson, 1933). Specific components of the bean undergo different chemical changes during fermentation.

The water content of the bean increases during fermentation from 33% moisture to 40% (Howat, 1957). This suggests among other things a movement of water from pulp into the bean but Howat (op.cit) showed that this was unlikely. Because the sugar content of the pulp is greater than that of the bean, a higher osmotic pressure is created in the pulp and water should move from the cotyledons to the pulp thus dehydrating the former. Howat (1957), therefore, suggested that the chemical breakdown of some constituents of the cotyledons with the production of carbon dioxide and water could be the cause of the increase in water content of the beans.

During fermentation, the fat droplets do not diffuse out of the cotyledons. Consequently, there is no change in the quantity or chemical composition of the cocoa butter. Also at this stage hydrolysis of sucrose occurs resulting in a decrease in amounts of non-reducing sugars. The products of hydrolysis diffuse from the cotyledons into the shell. Whilst there is no change in the content of starch, soluble amino acids are lost through diffusion into the testa. Amino acids that have been identified as occurring in cocoa beans are aspartic acid, glutamic acid, alanine, cysteine, leucine, serine, threonine, valine, proline and α-amino butyric acid (Maly, 1955; Forsyth and Quesnel, 1963).

The vitamin D concentration in the shell increases with fermentation and drying (Knapp and Coward 1934, 1935). These workers found the shell of sun-dried fermented cocoa from Ghana to contain 28 International Units (I.U.) of vitamin D per gram dry weight of testa tissue.

Plants react to invading parasites, through resistance mechanisms (Wood, 1967). These mechanisms may consist of structural or physical barriers to the invading process so that pathogens are excluded or killed locally to arrest further advance. Preformed inhibitors are widely recognised to be involved in disease resistance in some plant species. O-quinone, a toxic fermentation product of phenol oxidation in tea, makes the rolled tea uninhabitable to microorganisms (Lamb and Sreerangachar, 1940). Since the chemical constituents of cocoa beans are altered quantitatively and qualitatively during curing (Forsyth and Rombouts, 1951; Forsyth, 1952b), the natural protective action of cocoa bean constituents could be adversely affected. Laycock's (1931) observation that unfermented beans were immune to mould damage appears to reflect this view. While the ability of the non-volatile acids as disease inhibitors has not been investigated, it is established that the purine bases, particularly theobromine has no inhibiting effect on fungal growth. It is, in fact, used as a prefered source of nitrogen by micro-organisms. There is an observed significant fall in the level of the theobromine in the cotyledons with mould infection (Maclean, 1953; Roelofsen, 1958). In an eight-day fermentation trial, Humphries (1944) noticed a decrease in theobromine content of 20 - 24%. Similarly, Knapp and Wadsworth (1924) fermented beans for 10 days and showed that there was a movement of theobromine from the cotyledons into the testa. The longer the fermentation period, the greater the quantity of Theobromine that moved out of the testa. It was Roelofsen (1958), who described the diffusion of theobromine into the testa and its accumulation as crystals on the interior and exterior of the testa. These accumulations could be significant in increasing mouldiness by providing a ready source of nitrogen for invading fungi (Wadsworth, 1922).

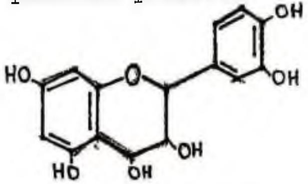
In contrast, phenolic compounds have been implicated in host defence mechanisms (Tomiyama, Sakai, Sakuma, Ishizaka, 1964; Kosuge, 1969). For a proper understanding of the role of phenolic compounds in cocoa beans it is necessary to obtain information on the changes in amounts of other chemical constituents such as protein, carbohydrates and organic acids so as to be able to ascertain whether decreasing amounts of these chemicals cause any noticeable effects (Kirkham, 1957). The potential of phenolic compounds and their oxidation products in host defence mechanisms have been amply demonstrated by Angell, Walker, Link (1930), Flood and Kirkham (1959) and Kiraly and Farkas (1961). Seevers and Daly (1970) however, failed to correlate resistance with phenolic compounds in the wheat stem rust and concluded thus; "It is becoming increasingly clear, however, that there is no single case where resistance and biosynthesis of aromatic compounds can be unambiguously correlated, let alone proven". They pointed out that resistance may be expressed through more subtle metabolic processes than by growth inhibition due to synthesised toxicants.

The phenolic compounds have a basic aromatic structure as the building units. The products of their enzymatic oxidation have been well investigated because of their role in chocolate flavour development (Forsyth, 1952b; Forsyth and Quesnel, 1957), and in other foods (Bate-Smith, 1954).

The bulk of the tannins and polyphenolic substances in the cotyledon are catechins and leucoanthocyanins which form 37% and 58% of the total tannin content, respectively. Of the soluble catechins 92% is (-)epicatechin (Forsyth, 1954). Apart from the catechins and leucoanthocyanins, the flavanoids occur in plants as glycosides in which some of the phenolic hydroxyl groups are combined with sugar residues. The sugar-free molecule is the aglycon. Hallas and Wight (1939) had reported that the percentage soluble tannins fell from 19% to 8% within 6 days of fermentation. Forsyth and Rombouts (1951) later found that the decrease was through exudation into the testa since the oxidases present were active only at the drying state when conditions were more aerobic. This idea had earlier been proposed by Humphries (1944) and later supported by Forsyth (1952). Rohan (1957) confirmed the presence of polyphenols in West African amelonado and determined their quantitative changes during fermentation. He found no changes in any of the phenolic compounds prior to death of the beans and the subsequent movement of the phenolic compounds from the storage cells. Following the movement of the compounds from the storage cells, however, that is about 12 hours after the beginning of fermentation, there is a rapid destruction of anthocyanins and a loss of the other polyphenolic compounds but at a much slower rate. The oxidation of the phenolic components of a mixture takes place in a sequence

(Roberts and Wood, 1950). As with tea fermentation, the sequence depends on the oxidation-reduction potential of the compounds. Thus in mixture of compounds, those with a lower oxidation-reduction potential are selectively oxidized. This observation was supported in the case of tea fermentation, by Kefford, (1959), and William, (1957). The order of oxidation, in a mixture, was suggested by these workers as catechol, pyrogallol, gallocatechin, then catechins, caffeic acid and chlorogenic acid. Quinones, formed from chlorogenic acid, appear to have a high oxidation-reduction potential and therefore tend not to be oxidized.

Again, as in the case of tea fermentation, Swain (1953) suggested that the various polyphenols are decomposed to the flavan-3: 4-diol form and then polymerised into insoluble fractions. Bate-Smith (1954) tried to correlate the flavanoid tannins with the basic flavan-3:4;diol as an intermediate of the hydrolyses of phenolic compounds. Roberts and Wood (1950) recognised the existence of this intermediate stage as the basic structure of all the flavanoids but they did not characterise it. Forsyth and Rombouts (1951) did thorough investigations and showed that the decomposition products of the major phenolic components are catechins, anthocyanins and leucoanthocyanins. They observed that the amount of total phenolic compounds remain unchanged. A degree of condensation occurred. The simpler polyphenols are destroyed and insoluble tannins formed. These workers also found that the flavan-3:4-diol was an inevitable intermediate in the decomposition and condensation of phenolic products.



flavan-3:4-diol

Some leucoanthocyanins are known to have flavan-3:4-diol units so that the structural similarity to the catechins became obvious.

Anthocyanins are hydrolysed by glycosidases present in the cotyledons of the beans (Forsyth and Quesnel, 1957). Some Aspergi-llus spp. produce the enzyme anthocyanase which hydrolyses anthocyanins to anthocyanidins (Nordstrom, 1956; Huang, 1955). The major part of the anthocyanin hydrolysis occurs during drying as free access to air leads to oxidation and browning (Forsyth and Quesnel, 1957). The rest is lost in storage with time (Kenten, 1965). It has been suggested that the action of glycosidases during the aerobic phase liberates a precursor of chocolate flavour (Forsyth and Quesnel, 1963).

The more complex leucoanthocyanidins are hydrolysed into (-)epicatechins and cyanidins (Bate-Smith, 1954; Forsyth and Roberts, 1960).

In general, Roberts (1957), Baruah and Swain (1959) have shown in the case of potato polyphenols, that flavonol glycosides, unlike their corresponding aglycons are not readily oxidized in the presence of potato phenolase except in coupled oxidations with substrates such as chlorogenic acid. It would appear from the claim of Roberts (1957), Baruah and Swain (1959) that anthocyanin glycosidases would be resistant to direct attack by phenolases. Indeed, Rohan (1957) detected residual anthocyanins in cocoa during the period of drying even though the phenolases were still presumably active. Some plants convert these phenolic compounds into compounds more toxic to the invading fungi (Schaal and Johnson, 1955). The ability of phenolic compounds to inhibit fungal activity can be reduced by the presence of proteins and certain amino acids, such as those containing sulphur, (eg. cysteine and glutathione which act as chelating agents (Roberts, 1957). Sporulation in *Venturia inaequalis*, the causal agent of the apple scab diseases is prevented by chlorogenic acid and iso-chlorogenic acid produced by the host plant (Flood and Kirkham, 1959). The onion rot of the yellow pigmented onion caused by *Botrytis* sp. and *Collectrotrichum circirans* is prevented by the production of catechol and protocatechuic acid (Link, Angell, and Walker, 1929a; Angell, Walker, Link, 1930; Link and Walker, 1933). Other workers investigating resistance mechanism of the Cox's pippin apple, observed that anthocyanins, leucoanthocyanins and flavans prevented its infection by *Geosporium perennans* (Byrde, Fielding and Williams, 1959; Hulme and Edney, 1959). The protective influence was found to be effective while the fruit was on the tree. At that stage there is a high phenolic level of 250 mg.

per 100g. dry weight of peel. Once the fruit is harvested, the concentration of phenolic compounds falls rapidly and at 20mg. per 100g. dry weight of peel, the fruits become more susceptible to the disease. Field investigations by Griffith (1958) on the effect of phenolic compounds in *Theobroma cacao* showed evidence of an intense metabolic activity involving the host polyphenols after invasion by *Marasmius permiciosus*. Also, while the fungus *Phytophthora palmivora* is able to infect and invade the entire pod within 14 days (Asare Nyako, 1972), its close co-member of the family Sterculiaceae and of the same environment, *Cola nitida*, is not affected. This would suggest intrinsic resistance mechanism in *C. nitida*.

At the beginning of the fermentation period, Roelofsen (1958) identified eight species of yeasts in the fermentation heap. These were *Saccharomyces cerevisiae* var *ellipsoides*, other *Saccharomyces* species, *Candida krusei*, *Kloeckera apiculata*, *Pichia fermentans*, *Hansenula anomala* and *Schizosacchromyces pombe*. After the first 36 hours, those yeasts requiring little oxygen, such as the *Saccharomyces* species, dominate. As the macerated pulp drains out, the conditions become more aerobic. Alcohol and acetic acid then become the main sources of carbon. At this stage, the aerophilic and thermotolerant yeast *Candida krusei* becomes the most important.

The initial concentration of citric acid in the pulp is high; thus producing a condition of pH 3.6 (Howat, 1957) which is favourable to *Betabacterium* species as the most significant lactic acid bacteria. In addition to *Betabacterium xylinum*, *Betabacterium xylinoides* and *Betabacterium ascendens*, *Acetobacter rancens* and *A. melanogenum* have been identified as the main acetic acid bacteria in fermentation heaps (Knapp and Coward, 1935; Roelofsen, 1958). *A. melanogenum* is known to be capable of removing pulp together with the outer epidermis of the seed coat, possibly by metabolic degradation of mucilage cells. *Candida krusei* has been observed in the cotyledons of cocoa from the Bahia region of Brazil and described there in visual examinations as internal moulds (Maravalhas, 1966).

Bunting's (1929a) investigation of internal mouldiness in commercially dry cocoa in Ghana revealed 5 fungal species as causal agents though their relative frequencies of occurrence were not stated. Members of the *Aspergillus glaucus* group, *A. fumigatus* and *A. ustus* and *Botryodiplodia theobromae* were identified. Since then not much work has been done in this area of cocoa quality control in Ghana.

The most recent investigations of internal moulds of Nigerian cocoa beans put the level at 1.6 per cent (Oyeniran and Adeniji, 1974). These workers identified 31 fungus species, belonging to thirteen genera, as the causal agents. These included twelve *Aspergillus* species and four *Penicillium* species. They grouped the fungi into major and minor pathogens depending on frequency of occurrence. The major pathogens were *Aspergillus chevalieri*, *Aspergillus flavus* *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus pernicilloides*, *Aspergillus ruber*, *Aspergillus tamarii*, *Botryodiplodia theobromae*, *Mucor pusillus*, *Paecilomyces varioti*, *Penicillium citrinum*, *Rhizopus arrhizus* and *Syncephalastrum racemosum*. The minor pathogens were listed as *Absidia corymbifera*, *Aspergillus aculeatus*, *Aspergillus nidulans*, *Aspergillus ochraceus*, *Aspergillus pseudoglaucus*, *Aspergillus restrictus*, *Curvularia lunata*, *Cylindrocarpon tonkinense*, *Fusarium oxysporium*, *Fusarium solani*, *Geotrichum candidum*, *Macrophoma* sp., *Penicillium decumbens*, *Penicillium steckii*, and *Penicillium variable*. The occurrence of *A. flavus*, which produces the mycotoxin, aflatoxin, among the list of fungi causing internal moulds of Nigerian cocoa, is of particular interest since Bunting (1929a) and Maravalhas (1966) did not find this species in their observations in Ghana and Brazil, respectively.

Reese and Downing (1951) and Olutiola (1976) have shown that *Aspergillus terveus*, *Aspergillus flavipes*, *Aspergillus fumigatus*, *Aspergillus fisheri*, *Aspergillus ustus*, *Aspergillus flavus* and *Aspergillus giganteus* have cellulolytic enzymes. With the notable exception of *Aspergillus niger* var *luchuensis*, the above observation agrees with the suggestion by Reese and Downing (1951) that no black Aspergillus attacks cellulose. Therefore, in the infection process, the fungi that can penetrate the cocoa bean could have ecological advantages and would pave the way for the entry of those that do not have cellulolytic enzymes and therefore cannot degrade cellulose walls (Reese and Levinson, 1925; Reese and Downing, 1951).

The method s by which fungi gain entry into the cotyledons of the bean are not fully known. Laycock (1931) suggested that fungi enter the bean through the micropyle end; the intact bean is invariably invaded at a point in the testa opposite the . radicle although there is no apparent structural difference between that part and other areas of the testa. Laycock (op.cit) showed that 76% of invasions of the cotyledons was through the radicle and 24% through other means. This report of the fungal penetration of the testa has been supported by Reese and Levinson (1925), de Witt (1952a) and Olutiola (1977). Renaud (1954) demonstrated that the point in the testa opposite the radicle is weakened during incipient germination, a condition encouraged by faulty fermentation procedure. Knapp (1937), on the other hand, noted that the testa is macerated by pectinases produced by yeasts during fermentation. Working in Bahia, Maravalhas (1966), however, observed that the raphe, which is also a scar tissue, rather than the micropyle, is the entrance point for invading fungi. It is the most porous part of the bean and is also the point through which pigments exude from the cotyledons and the entrance for acetic acid.

Phenolic compounds have been described as the antiseptics of plants by Pridham (1960). In the light of this claim, the ability of fungi to invade the cotyledons of cocoa beans, which contain phenolic compounds, generates a lot of interest. Various investigations have been undertaken to prove the potential of phenolic compounds as protectants against infection agents (Farkas and Kiraly, 1962; Kiraly and Farkas, 1962; Kuc, 1964).

The cocoa bean has a complex of enzymes responsible for browning, oxidation of phenolic compounds, and proteolysis (Huang, 1955; Forsyth, Quesnel and Roberts, 1958; Roelofsen, 1958; Holden, 1959). The other enzymes which have been found are B-fructosidase, B-glycosidase, B-galaclosidase, X-amylase, proteinase, asparaginase, phenoloxidase, peroxidase and catalase. However, an earlier report by Bavendamm (1928) claimed that no fungi occurring on cocoa produce phenoloxidases. Swain, (1953) observed, in the case of tea fermentation, that some products of enzymatic oxidation are able to precipitate protein and therefore enzymes. The more polymerised the products, the more they precipitate the enzymes. The process of phenol oxidation and subsequent polymerization of flavonols leads to a situation in which various enzyme systems can be precipitated out of action in the form of chelates and complexes (Chandler and Swain, 1959). Thus enzymatic reactions leading to flavour development could be hampered if the enzymes involved were precipitously inactivated (Swain, 1960; Jackson and Wood, 1959).

Besides the broad observation by Biodin and Prevost (1957) that some fungi need and indeed use tannins of cocoa beans in their growth processes, no work involving the action of specific phenolic compounds on the predominant moulding agents, the *Aspergillus* species, have been reported.

III. MATERIALS AND GENERAL METHODS

i) MATERIALS

Cocoa beans used in assessment studies were obtained from parcels of commercial cocoa beans in the warehouses of the Ghana Cocoa Marketing Board at the port of Tema from Oct. 1978 to. Random samples of 2 kg. weight were taken of the main season's crop and the light-season's crop of beans arriving at the warehouse from transit sheds. Ten samples were taken in all. The samples were stored at 4°C until required. Cocoa beans of the Amelonado type used in chemical analyses and for anatomical studies were taken from experimental fermenting heaps of the Cocoa Research Institute at Tafo in March 1979.

With the exception of Sclerotium rolfsii, isolates of all fungal species used in these studies, were prepared from infected beans collected from the Ghana Cocoa Marketing Board warehouses. The isolate of S. rolfsii was obtained from the stock culture collection of the Botany Department of the University of Ghana, Legon. This isolate was originally prepared from naturally infected tomato fruit (Lycopersicum esculentum) from Makola market in Accra.

ii) GENERAL METHODS

a) Maintenance of Stock Culture

Stock cultures of Aspergillus glaucus, A. niger, A. fumigatus A. tamarii, A. nidulans and S. rolfsii were maintained on Sabouraud's agar medium (40 g maltose; 10g peptone; 15g agar; 1000ml distilled water) in McCartney tubes. They were maintained at 25°C and subcultured at two-weekly intervals.

b) Method of Inoculation

For both liquid and plate cultures, a 3mm agar disc obtained from the growing edge of the culture was used for the inoculations.

c) Methods of Sterilization

McCartney tubes, pipettes, nutrient media and distilled water were sterilised by autoclaving for 15 minutes at 1.1 kg/cm^2 steam pressure and a temperature of 120°C. Cotton wool plugs, filter paper and pipettes were temporarily covered with grease paper to prevent the penetration of any condensed water during autoclaving.

Petri dishes (5cm and 9cm. diameter) were sterilised by heating at 165°C for 6 hours in an electrically heated oven.

Slides and coverslips were thoroughly washed with detergent, rinsed under running tap and in several changes of distilled water and stored in 90 per cent ethanol and flame-sterilised just before use. Inoculating needles and loops were flamed to red-heat and cooled immediately before use.

Cocoa beans were thoroughly washed under running tap then surface sterilized by dipping them in 0.1 mercuric chloride for one minute and rinsing thoroughly with several changes of sterile distilled water.

Cocoa testa extracts were sterilised by filtration through a millipore filteration unit (Filter Holder Part 4, XX1004703) and a filter of pore size 0.22 um under suction from a vaccum pump (Type B.S. 2408 B56).

The inoculating room was always sprayed with dettol solution (Dettol 5ml; water 95 ml) 10 minutes before use.

d) Assessment of percentage internal mouldiness in Commercial beans

One hundred cocoa beans from samples obtained by quartering were examined for internal mouldiness. Beans were surface sterilised and then drained on sterile filter paper (Whatman's No. 1). The washed beans were aseptically halved to expose as much of the cotyledons as possible. The cotyledons were incubated at 25° ± 1°C on moist sterile filter paper (Whatman's No. 2) for 7 days. Assessment of growth was done on the 7th day. Three replicates of each treatment were examined.

The moisture content of the sample cocoa beans was determined by the oven drying method.

e) Assessment of mycoflora of cocoa beans

Infected beans from an earlier investigation were transferred to Sabouraud's, Czapek Dox, and Potato Dextrose agar media and the fungal species which grew out of the cotyledons were isolated into pure cultures. All fungal species were subcultured on Czapek Dox agar medium according to the method of Thom and Raper (1960) for complete identification. Fungi were identified under the low-power of a microscope and/oil immersion. All plates were incubated/high pow for 10 days before identification was done.

f) Culture of test fungi on nutrient media

Sabouraud's agar (15 ml) in a 9 cm diameter Petri dish was inoculated with a 3mm culture disc removed with a No. 3 cork borer from the growing edge of a 3 days old culture raised on Sabouraud's agar. The inoculum was placed at the intersection of two diameters. There were three replicates for each treatment.

For the liquid medium, part of the mycelium from a 3 day old 1.25% water agar was inoculated into 30 ml broth in Erlenmeyer flasks.

g) Assessment of growth of cultures on media

i) Solid medium

Growth rate on agar medium was assessed by measuring the diameter of the cultures along two diameters at right angles to each other at 24 hours intervals.

ii) Liquid cultures

To assess the amount of mycelium produced by the test fungi, 3 mm diameter plugs from the growing edge of 1.25% water agar culture was used per flask. Mycelium was harvested onto a previously weighed Whatman's No.2 filter paper, and dried to constant weight at 60°C. The filter paper containing the dry mycelium was then weighed after it had been allowed to cool in a desiccator.

h) Assessment of growth on cocoa bean testa extracts

i) Preparation of cocoa bean testa extracts

For assessing the effect of bean testa during curing on the production of mycelia, 25g of bean testa separated from the cotyledons were ground in a blender (Sunbean, Model XBL) for 4 minutes, strained with cheese cloth, made up to 500 ml and sterilised using millipore filtration (filter pore size, 0.22 um) *in vacuo*. Erlenmeyer flasks (250 ml capacity) each containing 20 ml media were used for all the cultures in extracts.

ii) Spore germination methods

Spores were obtained by carefully removing spores from agar plates of 3 days old cultures. For each germination test the density of spore suspension was strictly standardised to 400,000 per millilitre of suspension with the aid of haemocytometer. Spores were suspended in the appropriate extract and incubated at 25°C ± 1°C for varying periods of time.

i) Assessment of Spore germination

At the end of the desired incubation period, spore germination was determined by recording; (a) percentage total spore germination and (b) length of germ tubes. Germination assessments were done for testa extracts and the control. A nutrient medium of Potato Dextrose Agar "floodwater" was used as control. A fresh uninoculated PDA plate was flooded with 10 ml. sterile distilled water and allowed to stand for five minutes. The water was poured off and used in the preparation of spore suspension.

j) Investigation of stage during curing when contamination was observed in cotyledons

Beans used for studies on the stage in cocoa bean curing when contamination was observed were removed from a 155 kg. fermenting heap of Amelonado type cocoa beans at the fermentary of the Cocoa Research Institute at Tafo. Fifty beans were removed randomly at various stages of curing viz. fermentation and drying (0, 2, 6 and 12 days). The 6-day method of fermentation was used. The beans were immediately surface-sterilised in 0.1% mercuric chloride solution, rinsed at least three times in sterile distilled water and cut transversely into 5 equal sections. The sections were placed on a moist filter paper (Whatmans No. 2) in sterile petri dishes in an air blown sterile chamber (Pathfinder, Havant, England) at 25°C. Beans were incubated for 7 days and then examined for evidence of fungal growth.

Hyphae were cultured for complete identification.

k) Chemical Analyses

1. Preparation of fat-free powders for analyses

One kilogram of the cocoa bean testa separated from the cotyledons of the sample cocoa beans was dried in a ventilated oven (Gallenkamp, Model 1H - 100) at 40°C for 48 hours. The dry testa was ground into powder. About ten grams of the ground testa were defatted by shaking in 100 ml petroleum ether (b.p. 40 to 60°C) for 3 hours. The solvent was decanted and discarded. The precipitate was extracted with fresh solvent, thoroughly drained and the powder stored in the dark under vacuum in a desiccator.

2. Phenolic compounds

Three grams of the defatted testa powder were extracted with 50 ml. absolute methanol for 2 hours at 22°C in the dark with occasional shaking. The supernatant was decanted and the pellet was suspended in a fresh volume of methanol and the extraction repeated twice. After the third extraction the supernatants were combined, filtered through Whatman's No. 1 filter paper and evaporated to dryness at 60°C in a Buchi rotary evaporator (Rotavapor-El Glasapparatefabils, Flawil). This dried sample contained the free soluble phenols.

The amino acids and sugars were removed by resuspending the pellet in 20 ml. of methanol (50% v/v) for 1 hour and filtered through Whatman's No. 1 filter paper to remove insoluble material. The filtrate was discarded. The residual powders were extracted with 70 ml. methanol (50% v/v) under reflux with boiling water for 15 minutes to hydrolyse the complex phenols. A drop of toluene, was added to prevent fungal growth.

3. Estimation of Total free phenols

The free phenol content was determined using the Folin-Ciolcateau phenol reagent with caffeic acid (Kock-light Laboratory Ltd, 4064) as the standard. A calibration curve was prepared with serially diluted pure caffeic acid solutions as shown in Appendix A. To 0.5 ml. of the absolute methanol fraction (diluted 10 times) was added 10 ml. of 2.8 N sodium carbonate solution (29.7 g in 200 ml. water) and 3.0 ml. phenol reagent with shaking at intervals for 2 minutes. The optical density of the test solution was measured at 650 nm on a spectrophotometer (Pye Unicam S.P. 600 Series 2) against controls made up of the sodium carbonate and the phenol reagent and the sodium carbonate and distilled water.

TABLE i

Reaction mixture for the determination of free phenols

Reactants	Quantities Used		
	Experimental	Controls	
		1*	2+
Test Solution	0.5 ml	0.5 ml	-
H_2O	4.5 ml	14.5 ml	5.0 ml
2.8N Na_2CO_3 Soln.	10.0 ml	-	10.0 ml
Phenol reagent++	3.0 ml	3.0 ml	3.0 ml
	18.0 ml	18.0 ml	18.0 ml

*Control 1 is for zeroing the photometer since there is no reaction in the absence of sodium carbonate.

+Control 2 is the effect of reagents in the absorption of the photometer. It is substracted from the readings of the experimental.

++Folin-Ciolcateu Phenol reagent: details of composition (Spies, 1965).

4. Estimation of Orthodihydric phenols

Orthodihydric phenol content of the testa was determined with Arnow's reagent using chlorogenic acid as the standard (Johnson and Schaal, 1952). The reaction mixture was made up of 0.2 ml extract (diluted 5 times), 0.2 ml. 0.5N HCl, 0.2 ml. of Arnow's reagent and 0.4 ml. of N NaOH in a total volume of 3.0 ml. The reaction was started by adding the Arnow's reagent and within

30 seconds the optical density (OD) was read at 520 nm against a blank in which water replaced the Arnow's reagent.

A calibration curve was prepared with serially diluted pure chlorogenic acid (Koch-Light, Laboratory Ltd, 40550) from a stock solution of 200 µg/ml and used as indicated in Appendix B.

TABLE ii

Spectrophotometric estimation of Orthodihydric phenols

Reactants	Experimental (ml)	Control (ml)
Test extract	0.2	0.2
H_2O	2.0	2.2
0.5N HCl	0.2	0.2
1N NaOH	0.4	0.4
*Arnow's reagent	0.2	-
	3.0	3.0

*Reactions were started by the addition of the Arnow's reagent (10g $NaNO_2$, 10g Na_2MoO_4 in 100 ml H_2O).

5. Flavonol determination by the vanillin method

The vanillin method of flavonol determination was used with (-)epicatechin (Koch-Light Laboratory, Ltd 40648) as standard (Swain and Hillis, 1959). One ml. of the extract was diluted 20 times and 2.0 ml. of the diluted extract used for the determinations.

Vanillin reagent (Hopkins & Williams Ltd. Batch 77314) was used to start the reactions. A calibration curve was prepared with serially diluted (–)epicatechin solutions as shown in Appendix C. Optical density readings were taken at 500 nm.

TABLE iii

Vanillin method for the determination of flavanols*

Reactants	Reactant Quantities (ml)		Blanks (ml)	
	A	B	C	D
Extract	0.2	0.2	-	-
Water	1.8	1.8	2.0	2.0
70% (v/v) H_2SO_4	-	4.0	-	4.0
+ Vanillin reagent	4.0	-	4.0	-
	6.0	6.0	6.0	6.0

For the determination OD readings produced by the reaction of the flavanol component, the OD readings of C and B were substracted from A. D is used to zero the spectrophotometer.

+ Reactions were started by the addition of Vanillin reagent.

(1 g vanillin in 100 ml. 70% (v/v) conc. H_2SO_4)

6. Anthocyanin determination

The determination of anthocyanins and leucoanthocyanin content was made using cyanidin chloride (Koch-light Laboratory, 40204 as the standard (Swain & Hillis, 1959). The cyanidin chloride was dissolved in 0.5N methanolic acid and a calibration curve prepared with serially diluted cyanidin chloride solutions as shown in Appendix D.

The stock extract (diluted 20 times) was used for the determinations and optical density readings measured at 525 nm against the blank.

TABLE iv

Determination of anthocyanin concentration

Reactants	Experimental (ml)	Blank (ml)
Extract	1.0	1.0
*Aqueous methanolic acid	3.0	3.0
+Methanolic HCl	-	1.0
●Hydrogen peroxide	1.0	-
	5.0	5.0

*Aqueous methanolic acid (0.5N HCl; 85% v/v absolute methanol).

+Methanolic hydrochloric acid (5:1 v/v aqueous methanol, 3N HCl)

●The reaction was started by the addition of hydrogen peroxide (1.0 ml freshly prepared 30% hydrogen peroxide in 9 ml. methanolic acid).

1) Culture media

Erlenmeyer flasks (250 ml capacity) each containing 30 ml of medium were used for all the liquid cultures. Each of the 9 cm diameter, petri dishes contained 20 ml. of agar medium.

1. **Potato Dextrose Agar:** 200g peeled Irish potato were boiled in 500 ml. distilled water, strained and made up to 1000 ml; 20g dextrose; 20g agar.

2. **Sabouraud's Agar:** 40 g maltose; 10 g peptone: 15 g agar in 1000 ml. distilled water.

3. **Czapek Dox Agar:** 2.0 g Sodium nitrate ($NaNO_3$); 1.0 g potassium phosphate (K_2HPO_4); 0.5g potassium chloride (KCl); 0.5g magnesium sulphate ($MgSO_4.7H_2O$); 0.01g ferrous sulphate ($FeSO_4$); 30g sucrose; 15.0 g agar; and 1000 ml ml distilled water.

4. **Sabouraud's broth medium:** 40g maltose; 10 g peptone; and 1000 ml. distilled water.

5. **Czapek Dox broth medium:** 2.0 g Sodium nitrate ($NaNO_3$); 1.0 g potassium phosphate (K_2HPO_4); 0.5g potassium chloride (KCl); 0.5g magnesium sulphate ($MgSO_4.7H_2O$); 0.01g ferrous sulphate ($FeSO_4$); 30g sucrose; and 1000 ml. distilled water.

m) Chemicals

All chemicals used in the preparation of media were either of the "Analar" or the "BDH" (British Drug House) grade.

n) Chromatographic analyses

1. Paper Chromatography

For the separation, detection and identification of phenolic compounds, the non-hydrolysed (absolute methanol) and the hydrolysed (50% methanol) fractions of the cocoa bean testa, were used in a one dimensional ascending chromatogram in a Shandon "UniKit" tank at 25° ± 1°C. The chromatograms were developed in two separate solvent mixtures: (i) BAW (n-butanol-glacial acetic acid, water, 4:1:5, top layer) (Griffith 1958); (ii) Forestal (acetic acid - conc. HCl-water, 10:3:30), 50% HoAc (50% aqueous acetic acid), BAW, PhOH (Phenol saturated with water), and water (Harborne, 1973).

Application of test Solutions and their detection

Extracts of (0.1ml) were spotted on chromatographic paper (Whatman No.1) by repeated application and dried in a cold stream of air from an electric hair-drier. Each chromatogram was run for 10 hours. At the end of this period, the paper was carefully removed from the tank, the margin of the solvent front marked, and the chromatogram dried in air. Standard markers of known identity were always run with the unknowns.

The dried chromatograms were detected by their fluorescence in short wave length (253 nm) and long wave length (365 nm) of ultra violet light from an Ultra Violet lamp (Chromato-Vue, Model cc 20) according to the recommendations of Griffith (1958). Further detection of the catechins and leucocyanidin was achieved by the use of chromogenic spray reagent diazotised p-nitroaniline (Swain, 1953).

2. Thin Layer Chromatography

For further study of the phenolic constituents of cocoa bean testa, the hydrolysed and non-hydrolysed extracts were separated by thin-layer chromatography (TLC) on plates (20 x 20 cm) of activated silica gel G (Type 60) containing fluorescent indicator and cellulose MN 300 (1:1) with a thickness of 0.25 mm. The extracts were spotted on plates, steamed and developed at room temperature 25° ± 1°C in the solvent system of chloroform-acetic acid-water (4:1:5) (CAW) as described by van Sumere et al (1965).

Identification of the spots was achieved by comparing the Rf values and colour reactions obtained by the use of a chromogenic spray reagent diazotised p-nitroaniline as described by Swain, (1953) with those of authentic phenolic markers. The spots were scraped individually and the phenolic compounds eluted from the silica gel by washing with ether. The extracts were centrifuged and the supernatant evaporated to dryness. The crystals formed were stored in a desiccator for subsequent experiments.

Confirmation of phenolic identifications by U.V. spectral comparisons was however, not done.

p) <u>Experimental precautions</u>

1. Glassware was kept scrupulously clean. Glassware which had already been cleaned with water and detergent was rinsed several times with tap water and three times with distilled water and allowed to drain before use.

2. Oven dried filter paper usually lost some weight because of heating. Filter paper used throughout these investigations was, therefore, heated at 75°C for 24 hours prior to use.

3. Filter paper with loads of dried mycelium were always conveyed to the balance room in a closed desiccator to avoid absorption of moisture.

4. Petri dishes with beans on agar plate media for mycoflora determinations were stored in platforms with legs standing in oil to prevent invasion by insects and other organisms.

5. Conidia of the same age were used in all experiments.

q) <u>Statistical methods</u>

Results were statistically analysed where appropriate.

IV. RESULTS

A. LEVEL OF FUNGAL CONTAMINATION OF COCOA BEANS AT TEMA

In 1930, cocoa from Ghana arriving at United Kingdom ports were found to contain 3.1 per cent fungal contamination or internal moulds (Passmore, 1932). Because of the absence of any more recent records, the level of contamination of cocoa beans, stored in the warehouse of the port of Tema, by fungi was investigated.

Cocoa bean samples were removed at monthly intervals from October 1978 to July 1979 as they arrived at the warehouse. The beans had been stored for varying periods at the various purchasing stations in the cocoa growing areas before arriving at Tema. There were neither records of the exact periods of storage at the different purchasing centres nor the conditions under which they were kept. The beans at the Tema warehouses also contained batches of both main crop, harvested in August to December in 1978, and the minor crop, harvested in May and June in 1979. Samples from the main and minor crops were kept separate.

Beans for the mycoflora studies were obtained by removing a handful of beans from the top, and then mid-section and bottom of every hundredth bag. The three lots from all the bags sampled were pooled together. Two kilograms of beans were finally taken on each sampling day and stored in sealed kilner jars in a freezer at 4°C until required for examination. The moisture content of the beans was in the meantime determined by drying samples from the remaining beans at 105°C in a ventilated oven for 3 hours.

One hundred beans were randomly selected from those stored in the freezer, surface-sterilised with 0.1% mercuric chloride solution and incubated at 25°C in humid chambers for 10 days. The percentages of beans containing fungi in the various testa are presented in Table 1. Each value in the Table represents the mean of three replicates of 100 beans each.

The results show an annual average fungal contamination of 4.9 per cent. The main crop had a lower average percentage of 3.9 per cent than the minor crop with an average contamination of 6.4 per cent.

There was, as should be expected, a relationship between moisture content of the beans and the levels of fungal contamination. Percentage contamination rose with increasing bean moisture content and fell with reduction in bean moisture content. (Fig. 1).

TABLE 1

MOISTURE CONTENT AND FUNGAL CONTAMINATION OF COCOA BEANS IN WAREHOUSES AT TEMA

CROP	YEAR AND MONTH OF SAMPLING (DATE OF ARRIVAL AT WAREHOUSE)	AVERAGE PERCENTAGE FUNGAL CONTAMINATION ± S.D.	AVERAGE PERCENTAGE MOISTURE CONTENT ± S.D.
MAIN	1978 NOVEMBER	6.0 ± 2.5	6.5 ± 1.9
	DECEMBER	2.3 ± 0.2	5.4 ± 1.6
	1979 JANUARY	3.7 ± 0.3	4.9 ± 2.0
	FEBRUARY	2.7 ± 1.4	5.7 ± 1.1
	MARCH	4.0 ± 2.5	6.3 ± 0.7
	APRIL	4.7 ± 1.9	6.0 ± 0.8
MINOR	1979 MAY	4.6 ± 1.9	7.5 ± 1.2
	JUNE	7.0 ± 2.2	7.3 ± 0.9
	JULY	8.0 ± 1.5	7.6 ± 2.1
	AUGUST	6.0 ± 1.4	6.3 ± 2.0

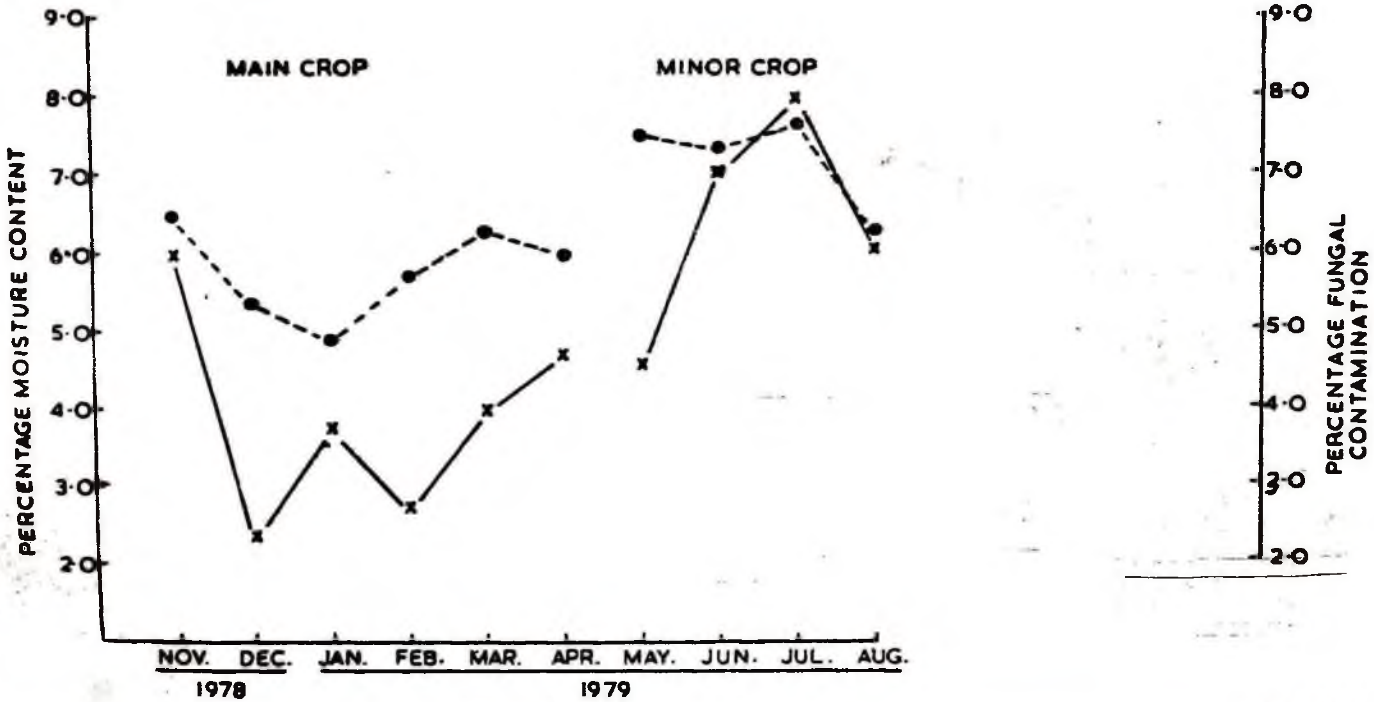


FIG.1 GRAPH SHOWING RELATIONSHIP BETWEEN MOISTURE CONTENT OF STORED COCOA BEANS (● - - - - - ●) AND THE LEVEL OF FUNGAL CONTAMINATION (X ——— X)

B. FUNGI ISOLATED FROM STORED COCOA BEANS

Dade (1929) and Bunting (1928, 1931) identified *Aspergillus fumigatus* and *Mucor buntingii* as regularly occurring in fermentation heaps. *Aspergillus flavus*, *Aspergillus tamarii* and *Aspergillus ochraceus* were isolated from dry cocoa beans by Bunting (1929a). In these reports the frequency of occurrence of these fungal contaminants was not shown. In Nigeria, as many as 31 fungus species have been identified as occurring in commercial cocoa beans (Oyeniran and Adeniji, 1974). It appears that the problem has not been fully examined in Ghana. Fungi, occuring in cocoa beans in the warehouses at Tema were therefore critically studied. Infected beans were plated on Potato Dextrose Agar, Sabouraud's Agar and Czapek Dox Agar media and fungi growing from them were recorded after 10 days.

Twenty-two fungi listed in Table 2 were isolated from the beans. These included three species: *Aspergillus phoenicis*, *Rhizopus oryzae* and *Neurospora crassa* being recorded for the first time on cocoa beans. The dominant contaminants were *Aspergillus chevalieri*, *Aspergillus tamarii*, *Neurospora crassa*, and *Penicillium citrium*. *Aspergillus ochraceus* which was isolated by Bunting (1931) was missing from the flora on beans in the Tema warehouse.

TABLE 2

FUNGI ISOLATED FROM STORED COCOA BEAN ON SABOURAUD'S AGAR

FUNGUS SPECIES	FREQUENCY OF OCCURRENCE %
Aspergillus chevalieri (Mang.) Thom & Church	17.6
A. flavus Link	5.3
A. fumigatus Fres	7.0
A. nidulans (Eidem) Wint	7.0
A. niger van Tieghem	2.6
A. pheonicis (Corda) Thom	2.6
A. restrictus Smith	7.0
A. ruber (Konig, Spieck & Brem) Thom & Church	1.0
A. sydowi (Bain and Sart) Thom & Church	0.3
A. tamarii Kita	13.0
A. ustus (Bainier) Thom & Church	0.3
A. wentii Wehmer	0.6
Botryodiplodia theobromae Pat.	3.6
Fusarium solani (Mart.) Sacc.	1.0
Mucor pusillus Lindt	8.6
Mycelia sterilia	2.6
Neurospora crassa Shear and Dodge	12.0
Penicillium citrium Thom	14.3
Penicillium sp . No. 1	4.3
Penicillium sp. No. 2	1.6
Rhizopus arrhizus Fischer	2.0
R. oryzae Went and Prinsen-Geerlings	8.3

C. EFFECT OF COCOA BEAN TESTA EXTRACT ON GROWTH OF SELECTED CONTAMINANT FUNGI

The influence of the host plant in host-parasite interaction is well known (eg. Tomiyama, Sakai, Sakuma, Ishizaka, 1964; Kosuge, 1969). It was therefore considered necessary to find the role of the testa in the invasion of the cocoa beans by fungi during storage. The influence of the bean on the invading fungi cannot be predicted in view of the existence of both stimulatory compounds, particularly, amino acids, carbohydrates and growth factors (Rohan, 1963;), and toxic compounds such as phenolic compounds and organic acids (Humphries, 1944; Roelofsen, 1958; Swain, 1960; Rohan, 1963). The growth of five _Aspergillus_ species, _A. flavus_, _A. fumigatus_, _A. nidulans_, _A. niger_ and _A. tamarii_, isolated from contaminated cocoa beans, and one non-contaminant _Sclerotium rolfsii_, in cocoa bean testa extracts was studied.

The extracts were prepared with testa taken from beans at varying stages of curing, and the dry weights of mycelia produced in these media after 7 days at 25°C are presented in Table 3a - f.

The results show an interesting response by fungi to the extracts. Growth was high in extract of testa of uncured beans in all cases. Extracts from 2- and 6- day cured beans were inhibitory, depressing growth of the fungi to varying degrees. While the extracts from beans cured for 8 and 12 days remained inhibitory to _A. fumigatus_ and _A. tamarii_, they supported approximately the same growth as extracts of testa of the uncured beans. These effects are clearly depicted in Fig. 2 which brings the data together for comparative purposes.

TABLE 3a

GROWTH OF ASPERGILLUS FLAVUS IN EXTRACT OF COCOA BEAN AT 25°C FOR 7 DAYS

Time of Curing of Bean [illegible] extract (Days)	Mean dry weight of mycelium (mg. ± S.D.)
0	45.6 ± 5.1
2	27.7 ± 6.2
6	30.4 ± 7.3
8	47.9 ± 1.9
12	44.7 ± 5.8

TABLE 3b

GROWTH OF ASPERGILLUS FUMIGATUS IN EXTRACT OF COCOA BEAN AT 25°C FOR 7 DAYS

Time of curing of Bean providing extract (Days)	Mean dry weight of mycelium (mg. ± S.D.)
0	64.3 ± 4.4
2	23.4 ± 7.2
6	25.0 ± 6.9
8	36.6 ± 2.3
12	43.3 ± 2.7

TABLE 3c

GROWTH OF ASPERGILLUS NIDULANS IN EXTRACT OF COCOA BEAN AT 25°C FOR 7 DAYS

Time of curing of Bean providing extract (Days)	Mean dry weight of mycelium (mg. ± S.D.)
0	46.9 ± 9.2
2	29.0 ± 6.4
6	31.5 ± 5.8
8	49.5 ± 2.3
12	48.1 ± 5.0

TABLE 3d

GROWTH OF ASPERGILLUS NIGER IN EXTRACT OF COCOA BEAN AT 25°C FOR 7 DAYS

Time of curing of Bean providing extract (Days)	Mean dry weight of mycelium (mg. ± S.D.)
0	43.9 ± 3.8
2	25.7 ± 6.1
6	26.8 ± 7.3
8	45.4 ± 2.7
12	41.3 ± 3.8

TABLE 3e

GROWTH OF ASPERGILLUS TAMARII IN EXTRACT OF COCOA BEAN AT 25°C FOR 7 DAYS

Time of curing of bean providing extract (Days)	Mean dry weight of mycelium (mg. ± S.D.)
0	69.2 ± 4.5
2	34.0 ± 4.0
6	28.6 ± 3.7
8	47.6 ± 2.7
12	43.1 ± 2.4

TABLE 3f

GROWTH OF SCLEROTIUM ROLFSII IN EXTRACT OF COCOA BEAN AT 25°C FOR 7 DAYS

Time of curing of bean providing extract (Days)	Mean dry weight of mycelium (mg. ± S.D.)
0	43.2 ± 6.6
2	40.9 ± 3.9
6	15.1 ± 4.8
8	56.2 ± 4.3
12	47.5 ± 2.4

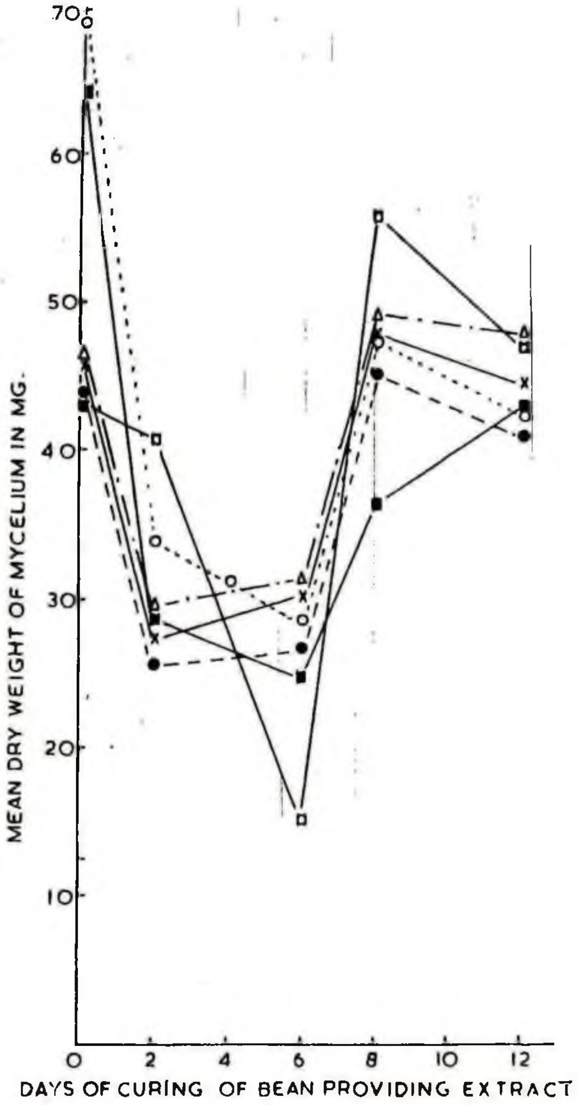


FIG 2 GROWTH OF FUNGI IN 20 ML COCOA BEAN TESTA EXTRACTS AT 25°C. FOR 7 DAYS.

X — X , *ASPEGILLUS FLAVUS*; ● ---● , *ASPERGILLUS NIGER*
■ —— ■ *A. FUMIGATUS*; O ··· O, *A. TAMARII*
Δ -··- Δ *A. NIDULANS*; □ — □, *SCLEROTIUM ROLFSII*

D. GROWTH OF THE FUNGI IN NUTRIENT MEDIA

A curious feature of the results of the experiments described in the previous chapter was the uniform pattern of response to the extracts of the cocoa testa by all the fungi tested. Besides, dry weights of mycelium produced in extracts of 8-day cured beans were practically the same. Two facts need to be established. First, are these fungi normally similar in their growth habits or the observations were the effects of the extracts. Secondly, do the observations indicate depressed or enhanced growth? The fungi were grown, in a subsequent experiment, in nutrient media in an attempt to provide answers to these questions.

The fungi were first grown in Czapek Dox Broth at 25°C and samples were drawn after 3, 7, 11 and 14 days, respectively, for dry weight determination of the mycelium produced. The results obtained are shown in Table 4. Each value is a mean of 4 replicates.

The results clearly show that the fungi varied in their growth habits. Differences were observed in (a) rate of growth, (b) time of attaining maximum growth and (c) the amount of mycelium produced. The last two features are shown very clearly in the graph in Fig. 3. Among the *Aspergillus* species, which came from cocoa beans, *A. tamarii* grew best, producing a maximum mean dry weight of 287.2 mg while *A. nidulans* provided the smallest mean maximum dry weight of 126.2mg.

A. fumigatus grew fastest in the medium, reaching maximum point of growth at either 3 days or between 3 and 7 days, followed by A. niger and A. tamarii which grew the greatest dry weights at 7 days, while maximum growth was not attained in A. flavus and A. nidulans until after 14 days.

Sclerotium rolfsii's pattern of growth was similar to those of A. niger and A. tamarii and was also next to these in productivity.

The experiment was repeated using Sabouraud's agar and broth media. There were four replicates for each species in each test. Tables 5 and 6 contain the results obtained in these experiments. The results can be summarised as follows: (a) there was variation in the amount of mycelium produced in Sabouraud's broth (b) comparing performance in the Czapek Dox broth and Sabouraud's broth after 7 days incubation, A. niger and A. tamarii grew less well in Sabouraud's medium, while A. nidulans was highly stimulated. (c) Growth rate varied on the agar medium, and (d) the poorest growth of A. nidulans in Czapek Dox broth among the species was confirmed by growth test on the agar medium at both 25° and 30°C.

TABLE 4

GROWTH OF ASPERGILLUS SPECIES ISOLATED FROM STORED COCOA BEANS AND SCLEROTIUM ROLFSII IN CZAPEK DOX BROTH AT 25°C

FUNGUS SPECIES	MEAN DRY WEIGHT OF MYCELIUM (mg ± S.D.) AFTER INDICATED DAYS OF INCUBATION			
	3	7	11	14
Aspergillus flavus	167.2 ± 4.7	168.2 ± 1.8	145.0 ± 4.0	186.2 ± 0.9
A. fumigatus	226.8 ± 8.4	199.5 ± 3.1	199.0 ± 7.6	158.4 ± 4.4
A. nidulans	73.2 ± 9.7	83.6 ± 3.4	107.1 ± 7.2	126.2 ± 5.7
A. niger	170.9 ± 10.4	279.2 ± 5.1	214.4 ± 6.1	218.9 ± 1.9
A. tamarii	154.2 ± 2.1	287.2 ± 4.0	247.3 ± 3.2	258.2 ±10.7
Sclerotium rolfsii	95.4 ± 0.8	277.2 ± 4.9	174.2 ± 8.3	196.5 ±14.5

TABLE 5

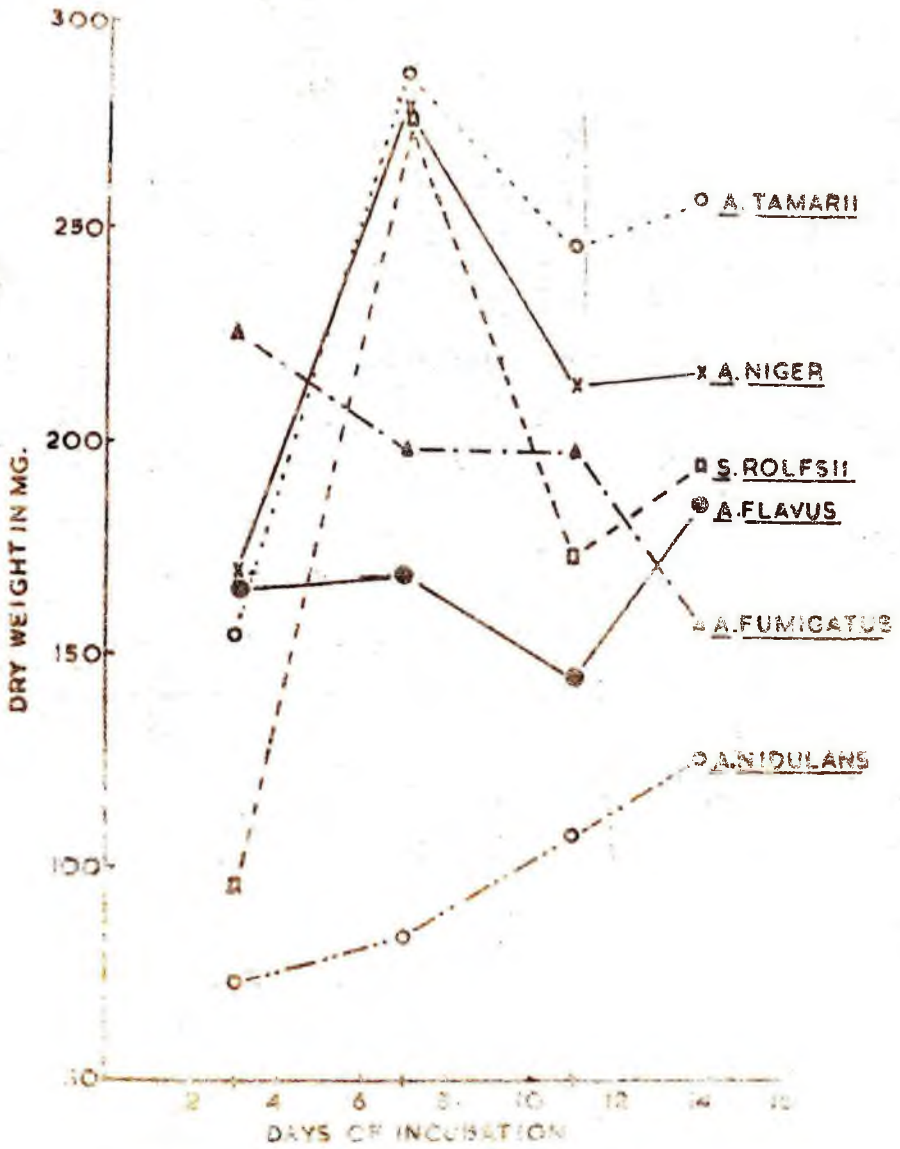
GROWTH OF *ASPERGILLUS* SPECIES ISOLATED FROM STORED COCOA BEANS AND *SCLEROTIUM ROLFSII* IN SABOURAUD'S BROTH AT 25°C FOR 7 DAYS

Fungus species	Mean dry weight of mycelium (mg. ± S.D.)
Aspergillus flavus	126.5 ± 9.1
A. fumigatus	230.2 ± 7.9
A. nidulans	172.1 ± 4.6
A. niger	129.3 ± 3.6
A. tamarii	124.0 ± 6.0
Sclerotium rolfsii	182.1 ± 5.0

TABLE 6

GROWTH OF ASPERGILLUS SPECIES ISOLATED FROM STORED COCOA BEANS AND SCLEROTIUM ROLFSII ON SABOURAUD'S AGAR MEDIUM AT 25° AND 30°C

Temperature of incubation (°C)	Fungus species	Mean diameter of colony (mm.) after indicated days of incubation 2	 4	 6	 8
25	*Aspergillus flavus*	11.0	25.0	37.0	51.5
	A. fumigatus	13.0	18.5	42.0	54.0
	A. nidulans	3.0	9.5	13.5	18.0
	A. niger	10.0	23.0	32.5	44.0
	A. tamarii	9.0	26.0	31.0	41.5
	Sclerotium rolfsii	13.0	26.0	39.0	51.5
30	*Aspergillus flavus*	16.0	30.0	43.0	64.5
	A. fumigatus	13.0	25.0	39.0	61.0
	A. nidulans	0.0	10.0	13.5	19.0
	A. niger	19.0	34.0	44.0	59.0
	A. tamarii	16.5	29.0	39.0	54.5
	Sclerotium rolfsii	11.5	22.5	35.0	50.5



[illegible] GROWTH OF *ASPERGILLUS* SPECIES [illegible] STORED [illegible] AND *SCLEROTIUM* [illegible] ON COPRA [illegible]

E. ANALYSIS OF PHENOLIC CONSTITUENTS OF THE COCOA BEAN TESTA

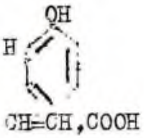
There is much published work on the chemical constituents of the cocoa bean (Forsyth, 1952b; Howat, 1957; Griffith, 1958; Swain, 1960; Rohan, 1963). Phenols are considered to be some of the important components of the cotyledons (Knapp, 1937; Forsyth, 1954; Forsyth and Quesnel, 1957; William, 1957; Forsyth and Rombouts, 1951; Roelofsen, 1958). In contrast, there is little published on the phenolic component of the testa even though Cadman (1959) considered the phenolic compounds of the cocoa bean testa to play a very significant role in the infection of the bean by pathogens. Reports available mostly contain qualitative rather than quantitative assessments of phenolic compounds of the testa. Studies of Forsyth and Rombouts, (1951) on quantitative changes showed that the total concentration of phenols in the bean remained unchanged during curing, whilst the simpler mono-phenols were destroyed and condensed into insoluble tannins and more complex phenols. In his analyses on the distribution of phenolic compounds in cocoa plants, Griffith (1958) observed that the testa contained leuco-anthocyanins not found in other parts of the plant. Leuco-anthocyanins are formed from the condensation of simpler phenols in the cotyledons which are then exuded into the testa. Analysis of the non-hydrolysed extracts of the testa showed that the only free soluble phenolic component of the testa was gentisic acid.

During the curing of cocoa beans, anthocyanins are destroyed after the first day of fermentation (Forsyth and Rombouts, 1951). Theobromine also leaches out of the cotyledons into the testa. Forsyth (1952b) demonstrated that as much as 20 - 30 per cent of catechins leached into the testa during fermentation.

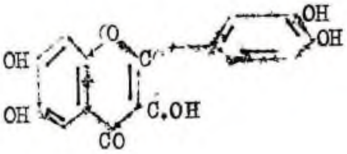
The amount of free phenols, orthodihydric phenols, flavonols, and anthocyanins in both the cocoa bean cotyledon and testa at different stages of curing were measured. There were two replicates for each test. Determinations made are presented respectively in Tables 7, 8, 9, 10 and in Fig. 4.

i) Free Phenols content

A wide variety of simple and complex compounds possessing phenolic hydroxyl groups occur in the cocoa plant tissue (Swain, 1960; Griffith, 1958) such as the simple caffeic acid and the complex flavonol.



Caffeic acid



flavonol (quercetin)

The simple or free phenols usually combine in polymerisation reactions to form complex insoluble tannins (Forsyth and Quesnel, 1957). Hallas and Wright (1939) showed that the concentration of soluble tannins fell during fermentation from a level of 19% of the total phenols to less than 8% at the end of fermentation. The results in Table 7 show that the initial concentration of free phenols was higher in the cotyledons than in the testa. The free soluble phenols broke down rapidly during fermentation and on the last day of fermentation only 0.73mg/g of the free soluble phenols was detected. With drying, the concentration reduced slightly to 0.70 mg/g. In the testa, the total free phenols concentration was lower than that of the cotyledons viz. 0.41 mg/g dry weight. This amount also fell with curing so that at the end of fermentation it had been reduced to 0.32 mg/g and to 0.22 mg/g on the 6th day of drying.

TABLE 7

FREE PHENOLS IN COCOA BEANS AT VARIOUS STAGES OF FERMENTATION AND DURING DRYING

Treatment of bean	Mean concentration of Free Phenols (mg/g ± S.D.)	
	COTYLEDON	TESTA
FERMENTATION		
0 DAYS	4.00 ± 1.11	0.41 ± 0.09
2 DAYS	1.13 ± 0.82	0.45 ± 0.08
6 DAYS	0.73 ± 0.16	0.32 ± 0.11
DRYING		
6 DAYS	0.70 ± 0.20	0.22 ± 0.06

ii) Orthodihydric Phenols content

The investigations of Adomako (1975) on the effect of orthodihydric phenols on virus diseases of the cocoa tree gave one of the few reports on the relationship between orthodihydric phenols and host-parasite interaction. Forsyth and Rombouts (1951) in discussing flavour development during fermentation, observed that there was some condensation resulting in the destruction of simple polyphenols and the formation of insoluble tannins. The elucidation of the flavan-3:4-diol as an inevitable intermediate in the hydrolysis of leucocyanidins supported this claim (Robinson and Robinson, 1933). The rapid reduction in the amounts of free soluble phenols as observed in the previous experiments suggested the formation of insoluble tannins as products of condensation and polymerisation reactions. Changes in the concentration of orthodihydric phenols in the cocoa bean during curing were determined spectrophotometrically at 520 nm.

As shown in Table 8, the initial concentration of orthodihydric phenols in the testa of unfermented cocoa beans of 2.53 mg/g was far higher than the concentration of 0.33 mg/g in the cotyledons. However, with fermentation, the concentration of orthodihydric phenols in the cotyledons increased rapidly to approximately ten times the initial amount at the end of the curing process. This suggested that some polymerisation of the free soluble phenols had occurred during fermentation of the cotyledons. On the other hand, the concentration of orthodihydric phenols in the testa decreased slightly in the first two days of curing before rising again to a level higher than that of the uncured bean. The concentrations of the orthodihydric phenols in both the cotyledons and testa decreased during drying of the fermented bean to approximately the same level.

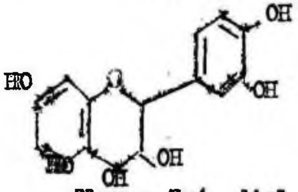
TABLE 8

ORTHODIHYDRIC PHENOLS IN COCOA BEANS AT VARIOUS STAGES OF FERMENTATION AND DURING DRYING

Treatment of bean	Mean concentration of orthodihydric phenols (mg/g ± S.D.)	
	COTYLEDON	TESTA
FERMENTATION		
0 DAY	0.33 ± 0.18	2.43 ± 0.22
2 DAYS	2.18 ± 0.47	2.19 ± 0.72
6 DAYS	3.41 ± 0.09	3.15 ± 0.81
DRYING		
6 DAYS	2.42 ± 0.88	2.46 ± 0.95

iii) Flavonol content

Polymerisation of free phenols and orthodihydric phenols would result in more complex flavonoid compounds such as flavonols being formed (Bate-Smith, 1954). These have the basic flavan-3:4-diol structure. Schrauffstater (1948) reported that morin, a derivative of flavonol



flavan-3:4- diol

showed a mild bacteriostatic effect on Staphylococcus aureus.

The concentration of flavonols in the cotyledons and the testa during curing were determined using the vanillin method (Swain and Hillis, 1959). The resulting yellowish colour was measured at 500nm (the wavelength at which absorbence was highest) with (-) epicatechin as standard.

There was a higher concentration of flavonols, according to the data in Table 9, in the unfermented testa (0.51mg/g dry weight) than in the cotyledons (0.21mg/g). During fermentation, while the flavonol concentration in the cotyledons increased to more than three times the initial amount at the end of the curing period, it decreased in the testa. The concentration then fell in both cotyledon and testa during the 6 days of drying.

TABLE 9

FLAVONOLS IN COCOA BEANS AT VARIOUS STAGES OF FERMENTATION AND DURING DRYING

Treatment of Bean	Mean concentration of flavonols (mg/g ± S.D.)	
	COTYLEDON	TESTA
FERMENTATION		
0 DAY	0.21 ± 0.25	0.51 ± 0.15
2 DAYS	0.39 ± 0.16	0.33 ± 0.14
6 DAYS	0.72 ± 0.09	0.43 ± 0.10
DRYING		
6 DAYS	0.49 ± 0.11	0.37 ± 0.04

iv) Anthocyanin content

Forsyth and Rombouts (1951) observed that anthocyanins were rapidly destroyed after the first day of fermentation. These compounds which form 4 per cent of total phenols in the unfermented bean (Forsyth and Quesnel, 1963) were responsible, together with leucoanthocyanins, for the browning of cocoa beans during fermentation.

Extracts of the cotyledon and testa were assayed spectrophotometrically at 525 nm after precipitation of other compounds which absorb at same wavelength (Swain and Hillis, 1959). Pure cyanidin chloride dissolved in 0.5N methanolic acid was used as a standard. The results of anthocyanin concentrations in cocoa bean during curing (Table 10) indicated a higher concentration in the cotyledons than in the testa of the unfermented beans. The amount in the cotyledons decreased at the onset of fermentation before rising again to a level above the initial concentration at the end of fermentation. The concentration decreased during drying. It was found that in the testa, the anthocyanin concentration increased throughout the period of fermentation and during drying.

TABLE 10

ANTHOCYANIN IN COCOA BEANS AT VARIOUS STAGES OF FERMENTATION AND DURING DRYING

Treatment of bean	Mean concentration of anthocyanin (mg/g ± S.D.)	
	COTYLEDON	TESTA
FERMENTATION		
0 DAY	0.16 ± 0.02	0.07 ± 0.02
2 DAYS	0.13 ± 0.02	0.07 ± 0.01
6 DAYS	0.20 ± 0.05	0.15 ± 0.04
DRYING		
6 DAYS	0.14 ± 0.01	0.24 ± 0.05

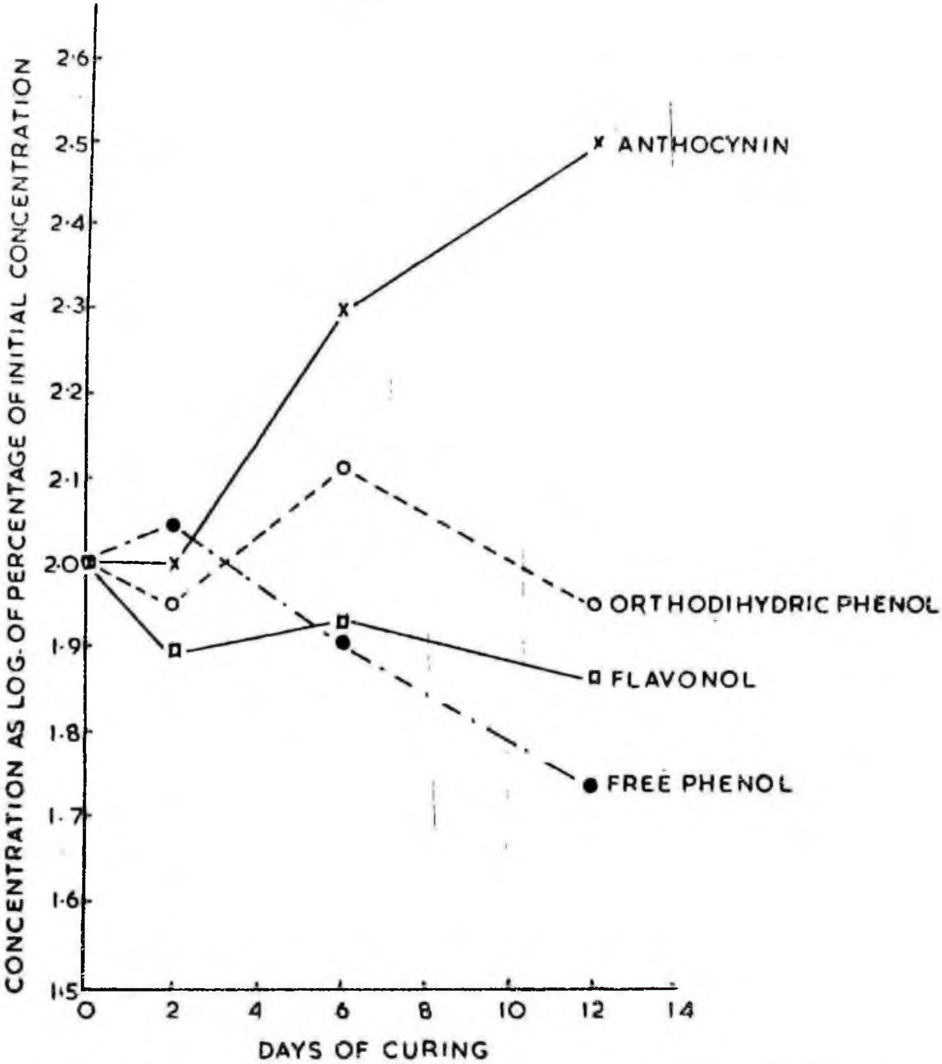


FIG. 4 PHENOLIC COMPOUNDS IN TESTA OF COCOA BEANS DURING CURING.

F. CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF PHENOLIC COMPOUNDS OF COCOA BEAN TESTA

Griffith (1958) identified by paper chromatography gentisic acid, cyanidin hydrochloride, epicatechins and anthocyanins in the testa of fresh cocoa beans. He also observed that phenolic compounds and other tannins especially salicyclic acid and cinnamic acid derivatives were germination inhibitors of spices and legume seeds. Caffeic acid, p-coumaric and protocatechuic acid, isolated from other plants, were found to be mild inhibitors (Mason, 1955; Varga and Koves, 1958; de Roubaix and Lazaar, 1960). These workers noted that no single phenolic compound ought to be considered a specific germination inhibitor since different combinations of all these substances occurred in different fruits. Forsyth (1952b) had earlier identified three anthocyanins in forestero cocoa; cyanidin monoglucose, cyanidin arabinoglucose and cyanidin diglucoside. While Yeboah (1965) achieved good separation of phenolic compounds of *Citrus aurantium* on paper chromatograms, Harborne (1973) reported that chromatographic separation of phenolic compounds was better on thin layer plates. The technique of thin layer chromatography (TLC) had also been used successfully by other workers (Stahl, 1969; Seikel, 1962, van Sumere *at al*.1965). Cocoa bean testa extracts were analysed by separation methods involving paper chromatography and thin layer chromatography. After extraction, the samples were spotted on Whatman's No. 1 chromatographic paper, the spots developed, and identified by their Rf values and colour reactions. Paper chromatograms of testa extracts (non-hydrolysed and hydrolysed) developed in two solvent mixtures (a) single solvent system, and (b) multi-solvent system are presented in Figs. 5a - d. Colour reactions of spots to short - and longwavelengths of UV light are shown on Plates 1a - d.

The pre-identified spots on the thin layer plates using Silica Gel G (Type 60) as support and development in chloroform-acetic acid-water (4-1-5) were collected separately and the phenolic components recovered by eluting in ether. Solutions eluted from these spots were centrifuged to remove the silica gel material and the supernatant was evaporated to dryness. The resulting crystals dissolved in distilled water were designated as NO_1 with Rf value of 0.42, $N_{(2)1}$ and $N_{(2)2}$ with Rf values 0.31 and 0.54 respectively. Crystals from spots of hydrolysed extracts of cocoa beans in the 2nd and 6th days of curing were recovered and designated $H_{(2)1}$ and $H_{(6)1}$ with Rf values 0.23 and 0.23 respectively. Drawn chromatograms of unhydrolysed and hydrolysed testa extracts separated by TLC are presented in Figs 5a - b and colour reactions shown in Tables 11e and 11f.

From charts of Rf values and colour reactions, only tentative identifications could be made since spectral studies were not carried out. There was better separation in the multi-solvent system

(Forestal; 50% Acetic acid; BAW; saturated phenol; and water) than in BAW (n-butanol-acetic acid-water, 4:1:5) alone (see Plate 1a and 1b). Separation of components was better on paper than on thin layer plates. Rf values of compounds ranged from 0.35 to 0.99.

There were more spots observed in unfermented testa extracts than in beans that had undergone some amount of curing (fermentation and drying) as shown in Plate 1a. The number of spots decreased with fermentation so that fermented beans in the 6th day of drying showed no observable spots. While in the unfermented beans 4 spots were observed in separation methods involving the single-solvent system, there were 3 spots on the second day of curing, one on the sixth day, and none on the sixth day of drying (see Plate 1a). Spots on the chromatograms of non-hydrolysed unfermented testa extracts were designated NU_1, NU_2, NU_3, NU_4 and NU_5 with increasing Rf values. The corresponding Rf values for the spots were 0.35, 0.48, 0.60 and 0.70, respectively, in n-butanol-acetic acid-water (BAW) and 0.58, 0.66, 0.88, 0.97 and 0.99 in the multi-solvent system. Spot NU_1 was pink in visible light and brown in UV light. These colours suggested that NU_1 could be an anthocyanin compound. However, the Rf value for the standard marker was 0.46 and so spectral studies would be necessary to confirm the chemical nature of the cyanidin compound. The Rf values of NU_3 and NSe_3 at 0.60 in n-butanol-acetic acid-water (BAW) were close enough to the standard marker (epicatechin) value of 0.66 to suggest that those spots were epicatechin derivatives.

When testa extracts were hydrolysed fewer spots were observed. While 4 spots were observed in non-hydrolysed unfermented beans, there were only 2 spots produced by hydrolysed unfermented beans when the chromatograms were developed in n-butanol-acetic acid-water solvent system. There was a similar trend with testa extracts in later stages of curing. The spots of hydrolysed unfermented beans were labelled HU_1, HU_2, HU_3 with Rf values of 0.35, and 0.57 respectively in n-butanol-acetic acid-water (BAW) and HU_1, HU_2, HU_3 with Rf values of 0.25, 0.45, 0.68 when developed in the multi-solvent system. No anthocyanin was observed in the chromatograms of extracts of testa from beans in the later stages of curing. From the Rf values of 0.35 and 0.57 for spots HU_1 and HU_2 for hydrolysed testa extracts of unfermented beans, it could be inferred that the aglycone could be malvidin which has an Rf value of 0.58 in n-butanol-acetic acid-water (BAW). Spots $HSix_1$ of testa extracts of beans in the sixth day of curing and spot HSe_1 of extracts of beans in the second day of curing possessed the same Rf value of 0.56 in n-butanol-acetic acid-water (BAW) and 0.44 in the multi-solvent system and could be the same compound.

TABLE 11a

COLOUR REACTION OF CHROMATOGRAM SPOTS OF NON-HYDROLYSED COCOA BEAN TESTA EXTRACT DEVELOPED IN SINGLE-SOLVENT SYSTEM

DAY OF CURING	CHROMATOGRAM SPOT	Rf	Colour Reaction			
			Visible Light	Ultra Violet Light		Diazotised p-nitroaniline
				Short wavelength	Long wavelength	
FERMENTATION						
0 DAY	NU_1	0.35	V	V	DULL BR	LY
	NU_2	0.48	LV	DULL BR	LY	Y
	NU_3	0.60	CL	FL WH	CL	LY
	NU_4	0.71	CL	CL	LY	CL
2 DAYS	NSe_1	0.35	LY	V	DULL BR	Y
	NSe_2	0.43	CL	CL	LY	LY
	NSe_3	0.60	CL	FL WH	CL	Y
6 DAYS	NSi_1	0.69	CL	BR	CL	LY
DRYING						
6 DAYS	-	-	-	-	-	-

KEY

V = Violet
LV = Faint Violet
CL = Colourless
Y = Yellow
LY = Pale Yellow
BR = Brown
DULL BR = Dull Brown
FL WH = Fluorescent White

TABLE 11b

COLOUR REACTION OF CHROMATOGRAM SPOTS OF NON-HYDROLYSED COCOA BEAN TESTA EXTRACT DEVELOPED IN MULTI-SOLVENT SYSTEM

DAY OF CURING	CHROMATOGRAM SPOT	Rf	Colour Reaction			
			Visible Light	Ultra Violet Light		Diazotised p-nitroaniline
				Shortwave-length	Longwave-length	
FERMENTATION						
0 DAY	NU$_1$	0.58	V	BR	BR	LY
	NU$_2$	0.66	CY	BR	DULL BR	Y
	NU$_3$	0.88	CL	DBL	FL BL-WH	LY
	NU$_4$	0.97	LY	FL BL	FL BL-WH	CL
	NU$_5$	0.99	CL	LBL	LY	CL
2 DAYS	NSe$_1$	0.61	LY	BR	LY	Y
	NSe$_2$	0.71	CL	BL	FL WH	LY
	NSe$_3$	0.86	CL	FL BL	FL BL-WH	CL
	NSe$_4$	0.91	CL	LBL	LY	CL
6 DAYS	-	-	-	-	-	-
DRYING						
6 DAYS	-	-	-	-	-	-

KEY

V = Violet
CL = Colourless
Y = Yellow

BR = Brown
DULL BR = Dull Brown
LY = Faint Yellow
BL = Blue

LBL = Light Blue
FLBL = Fluorescent blue
FL BL-WH Fluorescent bluish white
FL WH = Fluorescent white

TABLE 11c

COLOUR REACTIONS OF CHROMATOGRAM SPOTS OF HYDROLYSED COCOA BEAN TESTA EXTRACT DEVELOPED IN SINGLE-SOLVENT SYSTEM

DAY OF CURING	CHROMATOGRAM SPOT	Rf	COLOUR REACTION			
			VISIBLE LIGHT	Ultra Violet Light		Diazotised p-nitroaniline
				Short wavelength	Long wavelength	
FERMENTATION						
0 DAY	HU_1	0.35	CL	FL BL	FL WH	LY
	HU_2	0.57	CL	LBL	FL BL	CL
2 DAYS	HSe_1	0.56	CL	LBL	LY	CL
6 DAYS	HSi_1	0.56	CL	LBL	LY	CL
DRYING						
6 DAYS	-	-	-	-	-	-

KEY

CL = Colourless		FL BL = Fluorescent Blue	
LBL = Light Blue		FL WH = Fluorescent White	
LY = Faint Yellow			

TABLE 11d

COLOUR REACTION OF CHROMATOGRAM SPOTS OF HYDROLYSED COCOA BEAN TESTA EXTRACT DEVELOPED IN THE MULTI-SOLVENT SYSTEM

DAY OF CURING	CHROMA-TOGRAM SPOT	Rf	COLOUR REACTION			
			VISIBLE LIGHT	Ultra Violet Light		Diazotised p-nitroaniline
				Short wavelength	Long wavelength	
FERMEN-TATION						
0 DAY	HU_1	0.25	CL	FL BL	FL WH	LY
	HU_2	0.45	CL	LBL	FL BL	LY
	HU_3	0.68	CL	FL WH	FL WH	LY
2 DAYS	HSe_1	0.44	CL	LBL	FL BL	LY
6 DAYS	HSi_1	0.44	CL	LBL	FL BL	CL
DRYING						
6 DAYS	-	-	-	-	-	-

KEY

CL = Colourless

LY = Faint Yellow

LBL = Light Blue

FL BL = Fluorescent Blue

FL WH = Fluorescent White

TABLE 11e

COLOUR REACTION OF SPOTS ON THIN LAYER PLATES OF NON-HYDROLYSED COCOA BEAN TESTA EXTRACT DEVELOPED IN CHLOROFORM-ACETIC ACID-WATER

DAY OF CURING	THIN-LAYER SPOT	Rf	COLOUR REACTION			
			VISIBLE LIGHT	Ultra Violet Light		Diazotised p-nitroaniline
				Short wavelength	Long wavelength	
FERMENTATION						
0 DAY	$N(0)_1$	0.71	V	LY	DULLBR	Y
2 DAYS	$N(2)_1$	0.68	LY	DULLBR	BR	LY
	$N(2)_2$	0.90	CL	FL BL	FL WH	OR
6 DAYS	-	-	-	-	-	-
DRYING 6 DAYS	-	-	-	-	-	-

KEY

V = Violet

Y = Yellow

LY = Pale Yellow

CL = Colourless

BR = Brown

DULLBR = Dull Brown

FL BL = Fluorescent Blue

OR = Orange

TABLE 11f

COLOUR REACTION OF SPOTS ON THIN LAYER PLATES OF HYDROLYSED COCOA BEAN TESTA EXTRACT DEVELOPED IN CHLOROFORM-ACETIC ACID-WATER

DAY OF CURING	Thin-Layer Spot	Rf	Colour Reaction			
			Visible Light	Ultra Violet Light		Diazotised p-nitroaniline
				Short wavelength	Long wavelength	
FERMENTATION						
0 DAY	-	-	-	-	-	-
2 DAYS	$H(2)_1$	0.44	CL	FL BL	CL	LY
6 DAYS	$H(6)_1$	0.45	CL	FL BL	CL	LY
DRYING						
6 DAYS	-	-	-	-	-	-

KEY

CL = Colourless

LY = Pale Yellow

FLBL = Fluorescent Blue

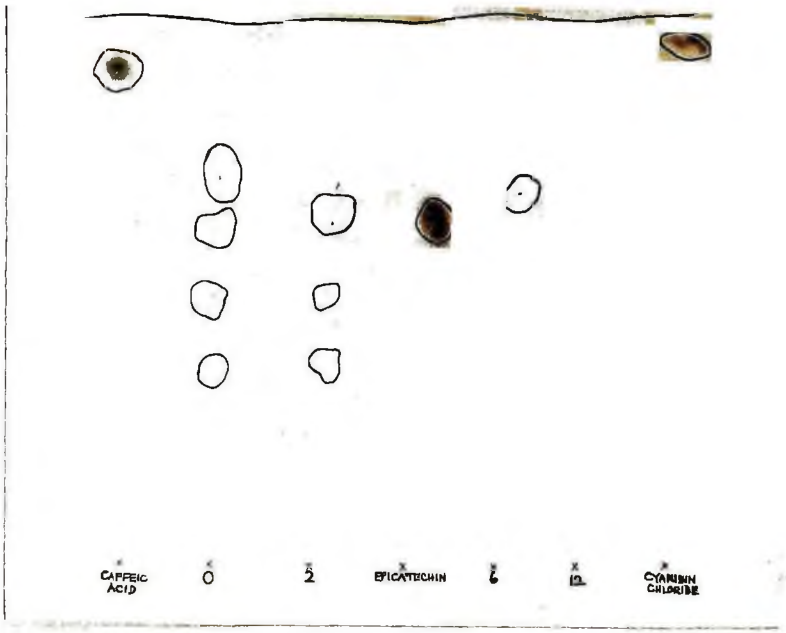


PLATE 1a: Photograph of chromatogram of phenolic components of nonhydrolysed (cocoa bean) testa extract on different days of curing, developed in single-solvent system.

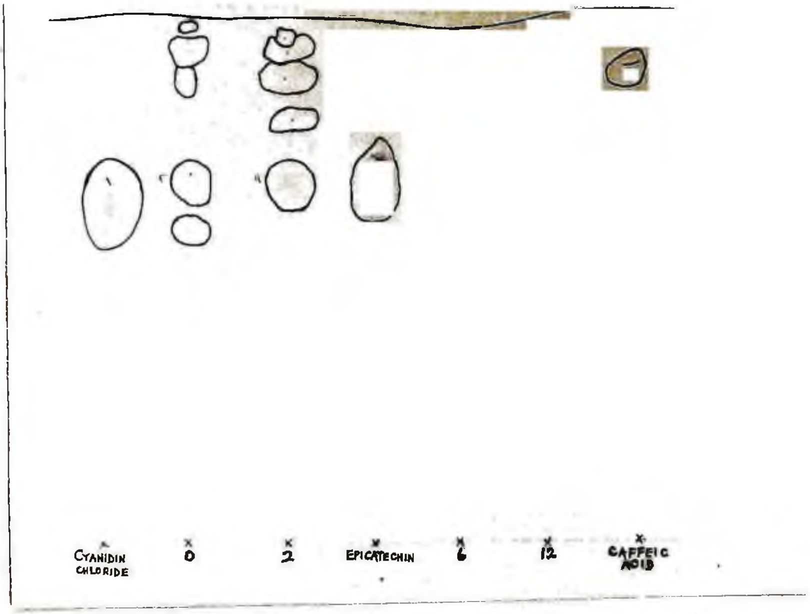


PLATE 1b: Photograph of chromatogram of phenolic components of nonhydrolysed (cocoa bean) testa extract on different days of curing, developed in multi-solvent system.

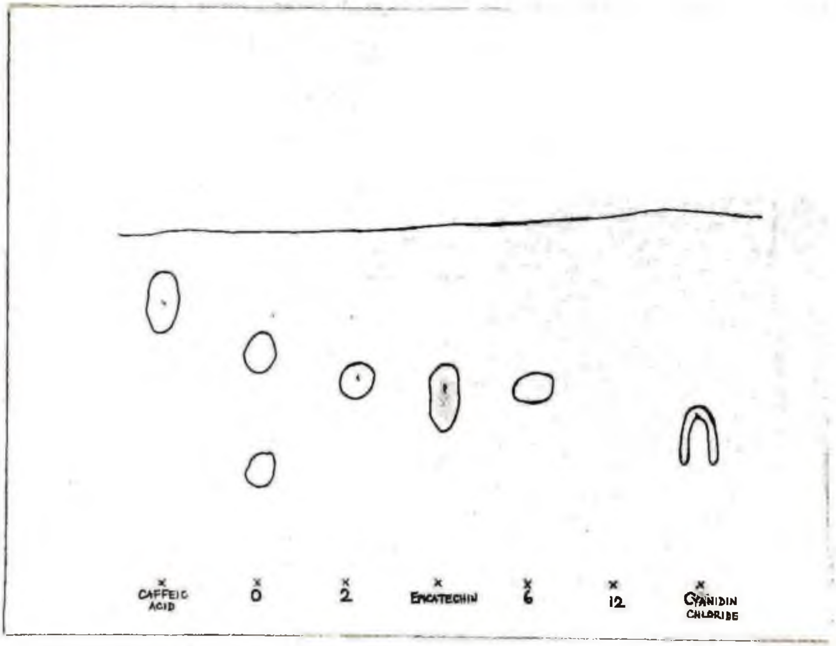


PLATE 1c: Photograph of chromatogram of phenolic components of hydrolysed (cocoa bean) testa extract on different days of curing, developed in single-solvent system.

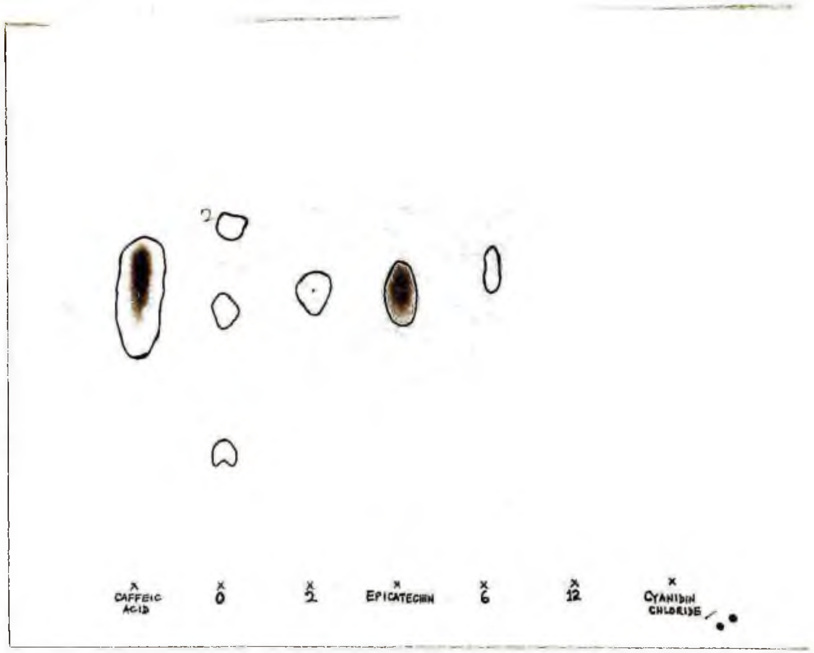


PLATE 1d: Photograph of chromatogram of phenolic components of hydrolysed (cocoa bean) testa extract on different days of curing, developed in multi-solvent system.

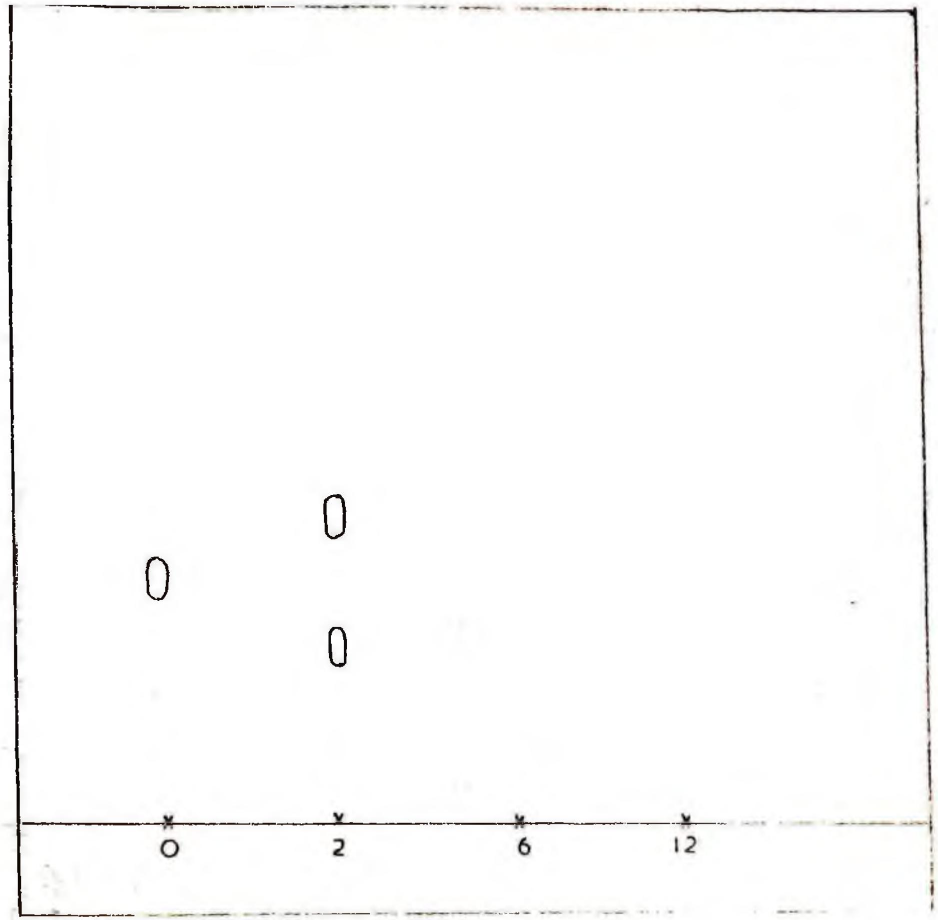


FIG. 5a DRAWING OF THIN LAYER PLATE OF PHENOLIC COMPONENTS OF NON-HYDROLYSED COCOA BEAN TESTA ON DIFFERENT DAYS OF CURING.

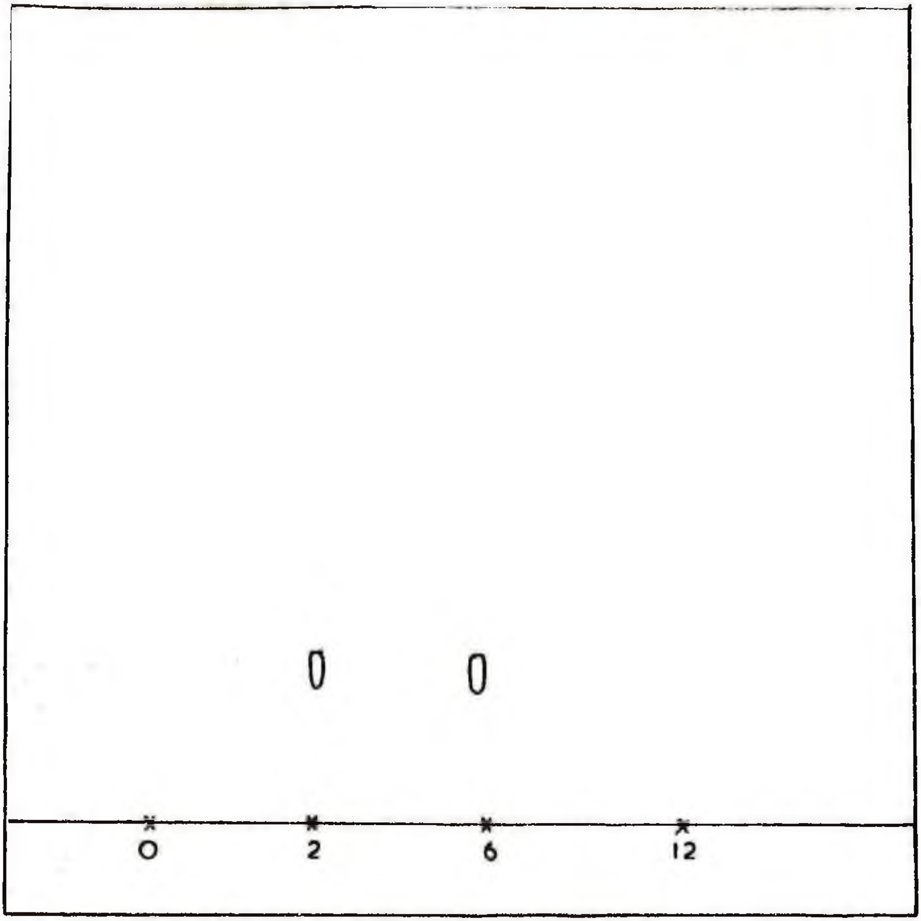


FIG. 5b DRAWINGS OF THIN-LAYER PLATE OF PHENOLIC COMPONENTS OF HYDROLYSED COCOA BEAN TESTA OF DIFFERENT DAYS OF CURING.

G. EFFECT OF TESTA COMPOUNDS OF ELUTED SPOTS OF TLC PLATES ON SPORE GERMINATION IN SELECTED COCOA BEAN CONTAMINANT FUNGI

1. Germination in "flood water" of Potato Dextrose Agar

The potential for infection of a pathogen depends on factors including the inoculum size and the germination characteristics of the spore. Spores of fungi which germinate readily in distilled water have sufficient nutrient reserve to support the initial germination process. There are ecological advantages if the endogenous nutrients occur in sufficient quantities to support initial growth of the germ-tubes. It is also advantageous to have an in-built system of discontinuous germination so that part of the inoculum can survive adverse conditions that may be lethal to the germinated spores.

Manners (1966) suggested that in assessing spore germination the measurement of three parameters best reflected the germination potential of fungal spores. These parameters are now very widely used in germination tests. The parameters are (a) the percentage total spore germination achieved within a certain time period. (b) the speed of germination or latent period of germination (ie. the time taken to produce 50 per cent spore germination) and (c) the amount of germ-tube growth or the yield which is a reflection of the amount of endogenous nutrient reserves.

In this investigation, percentage germination and germ-tube growth were studied in conidia of the four *Aspergillus* species used in previous experiments and the sclerotia of *Sclerotium rolfsii* using the methods of Manners (1966) and Dix (1972). Conidia of *Aspergillus* spp. do not normally germinate or do so very poorly in water (Lilly and Barnett, 1951). It would therefore be difficult to evaluate the effect of inhibitory compounds in subsequent studies using distilled water as control. Thus initial germination test was carried out with a dilute nutrient medium. A fresh uninoculated PDA plate was flooded with 10 ml of sterile distilled water and allowed to stand for five minutes. The water was poured off and used in the preparation of spore suspension. It was considered necessary to use this dilute nutrient medium which as the results in Table 12 show, was stimulatory enough to support germination. A very high nutrient concentration would overshadow the influence of weak inhibitory compounds or unduly reduce the effects of strong inhibitory compounds. Conidia and sclerotia were inoculated at 25° ± 1°C.

A. fumigatus germinated best among the *Aspergillus* species, with the remaining three showing percentage germination between 27 and 33 per cent in contrast to 71 per cent by *A. fumigatus*. Forty-one per cent of the sclerotia of *S. rolfsii* germinated in this medium. Germ tube growth was related to percentage germination. Among the *Aspergillus* species, *A. fumigatus* which germinated best had the longest germ tubes (mean of 7,110 μm).

There was positive correlation between final percentage germination and germ-tube length at $r = 0.20065$ and $P < 0.05$.

TABLE 12

GERMINATION OF CONIDIA OF *ASPERGILLUS* SPECIES AND SCLROTIA OF *SCLEROTIUM ROLFSII* IN EXUDATE OF PDA IN 50 HOURS INCUBATION AT 25°C

Fungus Species	Percentage Germination	Mean Germ Tube length (um ± S.D.)
A. flavus	27	3,125 ± 10.5
A. fumigatus	71	7,110 ± 7.8
A. niger	33	4,120 ± 14.6
A. tamarii	30	1,950 ± 9.9
S. rolfsii	41	10,250 ± 10.1

2. Germination of conidia and sclerotia in TLC eluted spots

Although phenolic compounds have been associated with disease resistance (eg. Hawley, Fleck and Richards, 1924), their influence on spore germination has not been extensively studied. Experiments were, therefore, carried out using *A. flavus*, *A. fumigatus*, *A. niger* *A. tamarii* and *S. rolfsii* to provide pertinent information. The spores of *Aspergillus*/and the sclerotia of *S. rolfsii* were germinated in solution of pre-identified spots on Thin Layer chromatographic plates in PDA "flood-water". Solution of eluted portions of the silica gel support without any compounds was used as a control. The samples tested were obtained from the following five chromatogram spots:

(a) First spot of unhydrolysed testa of unfermented cocoa bean $N_{(0)1}$.

(b) First and second spots of unhydrolysed testa of beans fermented for 2 days $N_{(2)1}$, and $N_{(2)2}$.

(c) First spot of hydrolysed extracts of beans fermented for 2 days $H_{(2)1}$.

(d) First spot of hydrolysed testa extracts of beans fermented for 6 days $H_{(6)1}$. Results of these investigations and data on control tests carried out with solutions of the silica gel support are given in Tables 13A - E.

The results show that both percentage germination and growth of germ tubes were affected by the compounds of the spots of the thin layer plates. The results are, briefly, as follows:

i. Compounds eluted from $N_{(0)1}$ (Table 13A) and $N_{(2)1}$ (Table 13B) depressed percentage germination of the species.

ii. With the exception of A. niger and S. rolfsii the germ tubes grew better in compounds of $N_{(0)1}$ than in the control (Table 13A). The depression in germ tube growth of those two was, however, very slight.

iii. Similarly, germ tube growth was varyingly affected by compounds eluted from $N_{(2)1}$ (Table 13B). Growth of germ tubes of A. flavus, A. niger and S. rolfsii was better, and that of A. fumigatus and A. tamarii was worse in compounds eluted from $N(2)_1$ than in the control medium.

iv. Compounds eluted from $N_{(2)2}$, $H_{(2)1}$ and $H_{(6)1}$ stimulated germination in some species and depressed germination in others (Tables 13C - E).

v. Percentage germination of A. niger was consistently stimulated by all the three compounds.

vi. Other instances of stimulation were, higher germination of A. flavus, A. fumigatus and A. tamarii in compounds eluted from $N_{(2)1}$ (Table 13C), and S. rolfsii in compounds eluted from $H_{(2)1}$ (Table 13D) and $H_{(6)1}$ (Table 13E).

vii. Germ tubes generally grew better in media which stimulated high percentage germination in the test medium and poorer in the inhibitory media. The exceptions were *A. flavus* in compounds eluted from $N_{(2)2}$ (Table 13C) and $H_{(6)1}$ (Table 13E) and *A. fumigatus* in compounds eluted from $H_{(6)1}$ (Table 13E).

TABLE 13A

Germination of conidia of *Aspergillus* species and sclerotia of *S. rolfsii* at 25°C for 50 hours in solutions of compounds eluted from TLC spots $R(0)_f$ of non-hydrolysed extracts of testa of unfermented cocoa beans.

Fungus Species	Germination in solution of compounds of eluted spots		Germination of solution of silica gel eluted spots	
	Percentage Germination	Mean germ tube length (µm)	Percentage Germination	Mean germ tube length (µm)
A. flavus	18	140	35	60
A. fumigatus	18	525	27	450
A. niger	39	200	49	250
A. tamarii	20	480	43	400
S. rolfsii	6.5	930	14	950

TABLE 13B

Germination of conidia of *Aspergillus* species and sclerotia of *S. rolfsii* at 25°C for 50 hours in solution of compounds eluted from TLC spot $N(2)_1$ of non-hydrolysed extract of testa of cocoa fermented for 2 days.

Fungus Species	Germination in solution of compounds of eluted spots		Germination in solution of silica gel eluted spots	
	Percentage Germination	Mean germ tube length (µm)	Percentage Germination	Mean germ tube length (µm)
A. flavus	27.5	475	32.5	225
A. fumigatus	20.0	300	27.0	600
A. niger	35.0	225	52.0	175
A. tamarii	24.0	350	47.0	500
S. rolfsii	8.0	1980	12.0	1950

TABLE 13C

Germination of conidia of *Aspergillus* species and sclerotia of *S. rolfsii* at 25°C for 50 hours in solution of compounds eluted from TLC spot $N(2)_2$ of non-hydrolysed extract of testa of cocoa beans fermented for 2 days.

Fungus Species	Germination in solution of compounds of eluted spots		Germination in solution of silica gel eluted spots	
	Percentage Germination	Mean germ tube length (μm)	Percentage Germination	Mean germ tube length (μm)
A. flavus	31	180	29	850
A. fumigatus	31	620	27	480
A. niger	23	160	21	180
A. tamarii	52	500	37	450
S. rolfsii	5	400	13	950

TABLE 13D

Germination of conidia of *Aspergillus* species and sclerotia of *S. rolfsii* at 25°C for 50 hours in solution of compounds eluted from TLC spot $H(2)_1$ of Hydrolysed extract of testa of cocoa bean fermented for 2 days.

Fungus Species	Germination in solution of compounds of eluted spots		Germination in solution of silica gel eluted spots	
	Percentage Germination	Mean germ tube length (µm)	Percentage Germination	Mean germ tube length (µm)
A. flavus	9	250	38	800
A. fumigatus	1	150	13	280
A. niger	33	250	17	140
A. tamarii	1	100	22	250
S. rolfsii	15	340	5	60

TABLE 13E

Germination of conidia of *Aspergillus* species and sclerotia of *S. rolfsii* at 25°C for 50 hours in solution of compounds eluted from TLC spot $H(6)_1$ of Hydrolysed extract of testa of cocoa bean fermented for 6 days.

Fungus Species	Germination in solution of compounds of eluted spots		Germination in solution of silica gel eluted spots	
	Percentage Germination	Mean germ tube length (µm)	Percentage Germination	Mean germ tube length (µm)
A. flavus	26	980	39	770
A. fumigatus	2.5	60	16	50
A. niger	35	410	17	140
A. tamarii	0	0	20	350
S. rolfsii	34	275	5	120

H. FUNGAL INVASION OF COTYLEDON OF COCOA BEANS DURING CURING

Fungi have been observed in cocoa beans at different stages of curing and storage. Dade (1929), Laycock (1931), de Witt (1961), Roelofsen (1958), Broadbent and Oyeniran (1967) and Wood (1967, 1979) have reported that fungi invade the beans at different stages of the fermentation process.

The history of the beans could sometimes influence their susceptibility to contaminant fungi. Dade (1927) demonstrated how pod diseases such as, blackpod caused by *Phytophthora palmivora* and mealypod caused by *Trachysphaera fructigena*, predisposed the beans to fungal invasion during fermentation and drying. He obtained no contamination when he fermented and dried beans from 100 healthy cocoa pods, but observed 30 per cent contamination when he fermented a mixture of beans removed from 50 healthy pods and 50 diseased pods. The pod pathogens might have caused sufficient damage to the testa to render it more penetrable by other fungi.

Very few studies have been devoted to the path of entry of the contaminant fungi. Laycock (1931) showed that 76 per cent of fungal contamination of the beans occurred through the micropylar end, and the rest through other points.

In this investigation, the occurrence of fungi in cocoa beans at different stages of fermentation and drying and the distribution of the fungi in the cotyledons were studied. Fifty beans were removed from the batch on each sampling date, they were surface-sterilised and asceptically cut transversely into 5 equal sections. The sections were incubated on moist sterile filter paper at 25° ± 2°C for 10 days. At the end of the incubation period, the fungi growing on the sections were studied and identified. The fungi identified and their distribution in the cotyledon are tabulated in Tables 14 and 15.

The fungal population increased with increase in time of curing. Regions of the bean nearest to the micropyle showed evidence of greater fungal invasion than other parts. Thus sections 4 and 5 contained greater total number of fungi than section 1, 2 and 3. *Aspergillus* was by far the dominant genus among the contaminants.

TABLE 14

Occurrence of fungi in beans of cocoa during curing

Days of Curing	Percentage fungal invasion of bean sections				
	1 (Stylar end)	2	3	4	5 (micropylar end)
FERMENTATION					
0	4	0	0	0	4
2	16	8	12	16	0
6	16	0	32	30	48
DRYING					
6	12	68	0	80	90

TABLE 15

Distribution of contaminant fungi in cotyledon of cocoa beans at varying stages of curing process.

Days of Curing	Fungus Species in bean Sections 1	2	3	4	5
Fermentation					
0	*Aspergillus* sp.				*Fusarium decemcellulare*
2	*Aspergillus restrictus*	*Aspergillus* sp	*Aspergillus fumigatus* *Mucor* sp.	*Mucor* sp.	
6	*A. restrictus* *A. fumigatus* *Mucor pusillus*		*Mucor* sp. *A. fumigatus*	*Rhizopus arrhizus* *A. fumigatus*	*A. fumigatus*
Drying					
6	*A. chevalieri* *Penicillium* sp. *A. tamarii*	*Mucor* sp. *Aspergillus* sp.		*A. flavus* *R. arrhizus*	*Mucor* sp. *Aspergillus* sp.

V. GENERAL DISCUSSION

A good chocolate aroma is of fundamental importance in the production of chocolate and chocolate related products. Anything which adversely affects this property is undesirable. Fungal contamination or internal mouldiness is the major cause of unpleasant odours and contaminations as low as 4 per cent could be detected in the taste of the chocolate products. Surprisingly, apart from the works of Dade (1929) and Bunting (1929), no record of the level of fungal contamination in Ghana cocoa is available even though fungal contamination of cocoa beans provides the main basis for grading of commercial cocoa beans.

The main aim of this study was to re-examine the mycoflora of cocoa beans, 50 years after the reports of Dade (op.cit) and Bunting (op.cit). The percentage of cocoa beans at the port of Tema contaminated by fungi was investigated for one cocoa season, October 1978 to July 1979. The average /4.9 per cent contamination recorded during this period fell within the world average range of 0 - 6 per cent (Dieckman, 1962) but considerably higher than the 1.6 per cent of Nigerian cocoa (Oyeniran and Adeniji, 1974), and slightly greater than 3.1 per cent recorded for Ghana cocoa arriving at the United Kingdom ports in 1960 (Passmore, 1932). Seasonal breakdown showed that the main crop cocoa contained an average 3.9 per cent (2.3 - 6.0 per cent) and the minor crop 6.4 per cent (4.7 - 8.0 per cent) fungal contamination (see Table 1 and Fig. 1).

The average moisture content was 6.5 per cent. This was lower than the minimum 7.5 per cent permissible in merchantable cocoa beans as recommended by the Working Party on Cocoa Grading. Percentage contamination was directly related to cocoa bean moisture content (see Table 1). Fungal contamination was higher during the rainy season and lower during the drier months of December to February.

Dade (1929) and Bunting (1929) isolated and identified five fungi belonging to the *Aspergillus* group, while Oyeniran and Adeniji (1974), working at the Stored Products Research Institute in Nigeria later found thirty-one spscies as occurring on Nigerian cocoa. Results of the present study showed that twenty-one fungal species belonging to nine genera were capable of infecting cocoa beans in Ghana. The list of the fungi observed and their frequency of occurrence is given in Table 2. Significantly, three fungus species *Aspergillus phoenicis*, *Rhizopus oryzae* and *Neurospora crassa* which had not been previously isolated anywhere on cocoa, were found. The results confirmed the suggestion by Reese and Downing (1951) that with the exception of *Aspergillus niger* var. *luchuensis* no black Aspergilli infested cocoa beans, and the observation by Bunting (1929) that the *Aspergillus glaucus* group (*A. chevalieri*, *A. ruber*, and *A. restrictus*) forms the most important contaminant of commercial cocoa beans.

Aspergillii constituted the dominant mycoflora of stored cocoa beans in both this study and in the work by Oyeniran and Adeniji (1974) on Nigerian cocoa. Consistently, *Aspergillus chevalieri* was the most frequently observed species in both cases. Also of particular interest is the occurrence of *A. flavus* (5.3 per cent) in this study and as a major contaminant in Nigeria although Bunting (1929) in Ghana and Maravalhas (1966) in Brazil did not find this species in their studies. In the reverse, *Aspergillus aculeatus* which was found in the beans by Bunting (1931) did not occur on beans examined in this study. It is not surprising that differences exist between the cocoa bean mycoflora of Ghana and Nigeria. *Paecilomyces varioti* and *Syncephalastrum racemosum* which were major contaminants of Nigeria cocoa as well as the minor species *Absidia corymbifera*, *Aspergillus pseudoglaucus*, *Curvularia lunata*, *Cylindrocarpon tonkinense*, *Fusarium oxysporium*, *Geotrichum candidum*, and *Macrophoma* species were absent in Ghana cocoa. On the other hand, Nigerian cocoa bean were free from *Neurospora crassa*, *Rhizopus oryzae*, *Aspergillus ustus*, *A. sydowi*, and *A. wentii* occurring in the beans in Ghana.

Notwithstanding the assurance by Wood (1979) that no aflatoxins had been detected in chocolate and chocolate products, the isolation of *A. flavus* (5.3 per cent) in mouldy cocoa beans made it necessary that further study of potential mycotoxin producing fungi and the characteristics of fungi associated with contaminated cocoa be more thoroughly investigated. Perhaps the absence of aflatoxin in cocoa is due to the unsuitability of cocoa bean as substrate for aflatoxin synthesis.

Temperatures as high as 51°C were reached on the third day of fermentation. Such high temperatures could kill most fungi except the thermophilic and thermotolerant species such as *A. fumigatus* and *Mucor pusillus*. Any fungus which entered the bean prior to fermentation would thus be killed during the process of fermentation so that most contaminants might have invaded the beans at the end of fermentation, [illegible] drying and in storage. During invasion, cellulolytic species notably *A. fumigatus*, *Botryodiplodia theobromae* and *Mucor pusillus* would be expected to play the role of pioneers creating access ways for the entry of non-cellulolytic species. Forsyth (1954) reported that cocoa beans with acceptable aroma could be obtained after only two days fermentation. If indeed most infection takes place after fermentation, the shortening of the fermentation process should have no influence on fungal activity in the beans in storage. Shortening of the fermentation period should be accompanied by measures to control fungal infection during storage and shipping of the beans.

The results of previous experiments showed that the *Aspergillus* species formed 64.3 per cent of fungi contaminating stored cocoa bean. Five *Aspergillus* species (*A. flavus*, *A. fumigatus*, *A. niger*, *A. nidulans*, and *A. tamarii*) isolated in this study from the contaminated beans were used for further studies, in comparison with *Sclerotium rolfsii*, a soil-inhabiting facultative parasite which is not native to cocoa beans.

The Aspergilli showed high mycelium production in testa extracts of unfermented cocoa beans. The greatest amount of growth was recorded in *A. tamarii* and *A. fumigatus* cultures which produced 69.2 mg and 64.2 mg dry weight, respectively, after 7 days of incubation (see Table 3b and 3c). The unfermented testa extract which contained much pulp is known to be high in carbohydrates especially the manoses (Forsyth and Quesnel, 1963). *S. rolfsii* produced the least amount of mycelium of 43.2 mg dry weight (see Table 3f). Extracts from 2- and 6-days cured beans depressed growth in all the fungi tested. The *Aspergillus* species were more inhibited than *S. rolfsii* by the extracts of 2-day cured beans. On the other hand, *S. rolfsii* was most severely inhibited by the extracts of 6-day cured beans (see Table 3f and Fig. 2). The respective percentage reduction in *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. tamarii* and *S. rolfsii* was 33.4, 63.6, 31.9, 39.8, 57.1 and 65.1 per cent. Alcohol, which is a major component of pulp at the beginning of fermentation as yeast activity increases, would be a significant constituent in the testa extract of cocoa beans (Roelofsen, 1958). The usual end-products of fermentation, acetic acid and lactic acid might be contributory factors to the observed depression of growth. Testa extracts of cocoa beans dried for 2 and 6 days after fermentation supported almost the same amounts of growth as the extracts of the unfermented beans. Growth was stimulated in *S. rolfsii* in extracts of beans dried for 2 days. Perhaps, oxidation of acetic acid, accumulated in the fermented beans, to carbon dioxide and water markedly reduced the level of acid content of the testa during drying.

The rate of growth in the extract media should be related to the intrinsic growth habit of the fungi. Dry matter produced by the various test fungi in Czapek Dox broth medium showed that the fungi varied in their growth habits. *A. fumigatus* grew fastest, attaining maximum growth on or about the third day of incubation in Czapek Dox broth medium at 25°C. In comparison it took 7 days in *A. tamarii*, *A. niger* and *S. rolfsii* (see Table 4 and Fig. 3). *A. nidulans* grew the slowest in Czapek Dox broth medium attaining maximum dry weight on the 14th day of incubation. It could be inferred that the amount of growth obtained in the previous studies using extract media reflected maximum growth in *A. niger*, *A. tamarii*, and *S. rolfsii* and not in the remaining species.

On Sabouraud's broth medium, *A. niger*, and *A. tamarii* grew less well. At 25°C *A. niger* produced 129.3 mg dry matter on Sabouraud's broth compared with 279.2 mg dry weight after 7 days incubation in

Czapek Dox broth. Correspondingly, *A. tamarii* produced 124.0 mg dry weight of mycelium in Sabourauds broth and 287 mg in Czapek Dox broth. *A. fumigatus* grew better in Sabourauds broth (230.2 mg dry weight) than in Czapek Dox broth (199.5 mg dry weight). The poorest growth of *A. nidulans* among the species tested in Czapek Dox broth was confirmed by growth tests in Sabourauds agar medium at 25°C and 30°C (See Table 4 and 6, and Fig. 3). This should not be surprising since the cultures were harvested as long as 7 days before reaching maximum growth. *S. rolfsii* which was next to *A. nidulans* and *A. niger* in productivity in Czapek Dox broth medium, grew better than both in Sabourauds broth.

Hall (1975) reported the presence of a germination inhibitor in the testa, and beans with testa removed germinated better than those with intact testa. He, however, did not characterise the chemical involved. It is possible that phenolic compounds present in the testa might be involved since these compounds have been identified as fungal growth inhibitors in other studies (Kirkham, 1957; Farkas and Kiraly, 1962; Kuc, 1964; Kosuge, 1969). In the present study, it was observed that there was a slight initial decrease in the total phenol concentration in the testa of cocoa bean with time during fermentation from 4.4 mg per gram to 3.1 mg per gram but rose thereafter during drying of the beans. Forsyth and Rombouts (1951) observed a general decrease in the phenolic compounds of the cotyledons during curing and suggested the fall to be either due to their destruction by enzymes or exudation into the testa. Results of this investigation tended to support the latter view.

The concentration of anthocyanin compounds in the testa increased sharply as soon as fermentation started from 0.073 mg per gram in the unfermented bean testa to 0.074 mg per gram, 0.15 mg per gram, and 0.24 mg per gram, in the 2nd 5th and 12th day extracts respectively. Kenten (1965), however, reported that in the case of Amelonado cocoa beans, the anthocyanin content decreased with time in storage. It is suggested that apart from the initial amount of anthocyanin compounds in the testa, more leached from the cotyledons into the testa.

Soluble phenols of the testa increased initially from 0.41 mg per gram to 0.45 mg per gram in the first 2 days of curing. Later the concentration fell to 0.32 mg per gram on the 6th day and then 0.22 mg per gram on the 12th day of curing. Cifferi (1931) suggested that since there was a large amount of oxidases present in the cocoa bean, an oxidation process might lead to the degradation of phenolic compounds resulting in their breakdown. Forsyth and Rombouts (1951), however, observed that by a process of condensation, the simpler soluble polyphenols were destroyed and insoluble tannins formed.

The orthodihydric phenols and the flavonols followed a similar pattern of an initial decrease in the amounts of these constituents at the beginning of fermentation and an increase during the later part of curing (see Table 7 and Fig. 4). The unfermented bean testa contained 2.43 mg per gram of orthodihydric phenols. On the second day, the concentration fell to 2.19 mg per gram of dry testa tissue. Forsyth and Rombouts (1951) suggested that this was either due to leaching or polymerisation of simpler leucocyanidins to more complex leucocyanidins. Similarly, flavonol compounds in the testa fell in amount with fermentation from 0.51 mg per gram in the unfermented bean to 0.33 mg per gram in two days of fermentation. Concentrations in the testa built up to 0.43 mg per gram at the end of fermentation. Again during drying, there was a fall in the amount of flavonols to 0.37 mg per gram of testa tissue.

It was suspected that there would be a relationship between these changes and the chemical factors involved in resistance to fungal invasion of plants and plant products. Further study of the chemical constituents on paper and thin layer chromatograms was therefore done. The paper chromatograms revealed more spots than thin layer plates. On the paper chromatograms five spots were observed for unhydrolysed unfermented testa extracts while only one developed on thin layer plates (see Fig. 5a and 6a). Separation was better in the multi-solvent system of Forestal, 50% acetic acid, n-butanol-acetic acid-water, saturated phenol and water, than in the chromatograms developed in n-butanol-acetic acid-water (4:1:5) alone (see Fig. 5a and Fig. 5b). When developed in the multi-solvent system, the unfermented non-hydrolysed testa extracts revealed five spots as compared to three in the hydrolysed extracts (see Fig. 5b and 5d). Roberts (1957) explained that hydrolysis resulted in decarboxylation of phenolic compounds into the basic aglycones and the methyl and carboxyl groups. Also, non-hydrolysed testa of beans in 2 days of fermentation had three spots when developed in n-butanol-acetic acid-water while the hydrolysed extracts had one spot. Spots of unfermented non-hydrolysed testa extracts designated NU_1, NU_2, NU_3, NU_4 and NU_5 had Rf values of 0.58, 0.66, 0.88, 0.97 and 0.99 respectively in the multi-solvent system. Spot NU_1 was pink in visible light and brown in the short wavelength band of UV light and could be identified as an anthocyanin compound. Forsyth (1952b) had identified three anthocyanins in Forestero cocoa : cyanidin monoglucose, cyanidin arabinocyanins and cyanidin diglycoside. Two of these, cyanidin arabinocynins and cyanidin diglycoside were later confirmed by Kenten (1965).

Hydrolysed extracts of unfermented beans developed three spots HU_1, HU_2, HU_3 with Rf values of 0.25, 0.43 and 0.68 respectively in the multi-solvent system. In the n-butanol-acetic acid-water solvent system, HU_1 and HU_2 had Rf values of 0.35 and 0.57.

The Rf value of HU_2 was regarded close enough to the Rf value of 0.58 for malvidin in n-butanol-acetic acid-water to suggest that HU_2 could be a malvidin glycoside. Results of these investigations were not conclusive since spectral studies were not done and fuller investigation is necessary in any future relevant studies.

Spots of phenolic compounds on the thin layer plates were eluted and used in spore germination tests. Since conidia of the Aspergilli do not normally germinate in or do so poorly in distilled water (Lilly and Barnett, 1951), it would be difficult to identify inhibition by the solutions if distilled water was used as control. A preliminary germination test of the fungi under study using a dilute nutrient medium of Potato Dextrose Agar "flood-water" was found stimulatory enough to support germination (see Table 14). *A. fumigatus* germinated best producing 71 per cent germination after 50 hours incubation at 25°C. *A. niger*, *A. tamarii* and *A. flavus* were next with 33, 30 and 27 per cent germination respectively (see Table 12). Forty-one per cent of the sclerotia of *S. rolfsii* germinated in the medium.

As expected, the sclerotia had the greatest amount of nutrient reserves and *S. rolfsii* had the longest hyphae (mean 10,250 μm). Among the *Aspergillus* species, *A. fumigatus* which germinated best had the longest hyphae (mean 7,110 μm) and *A. tamarii*, the shortest (1,950 μm). Even though only 27 per cent of the conidia of *A. niger* had germinated after 50 hours incubation, the total length of germ tubes produced was higher than for *A. tamarii*.

The effects of solutions of the spots eluted from thin layer plates on the conidia of the Aspergilli and sclerotia of *S. rolfsii* showed varied responses. Compounds eluted from spot NU_1 (Table 13A) which had been tentatively identified as an anthocyanin compound and the solution of spot NSe_1 (Table 13B) generally depressed germination. With the exception of *A. niger* and *S. rolfsii*, germ tubes of the others grew better in compounds NU_1 than in the control (see Table 12A). In the NSe_1 solution, *A. flavus*, *A. niger*, and *A. fumigatus* and *A. tamarii* worse than the control. Compounds eluted from NSe_2 stimulated percentage germination in the *Aspergillus* species but depressed germination in *S. rolfsii* (Table 13C). With the exception of *A. fumigatus* and *A. tamarii* germ tube growth was depressed.

Compounds of spots from hydrolysed extracts (HSe_1) and (HSi_1) had the same effect on percentage germination. Their effects stimulated the germination of *A. niger* and the sclerotia of *S. rolfsii* but depressed all others; the severest effect being recorded on *A. tamarii* (99 - 100 per cent) and *A. fumigatus* (97.5 - 99 per cent). While HSe_1 tended to depress germ tube growth among the *Aspergillus* species, with the notable exception of *A. niger*. HSi_1 uniformly stimulated growth of the germ tubes and hyphae of *S. rolfsii*.

These observations point out that, uniform effects of the phenolic compounds in the testa of cocoa beans on the contaminant fungi should not be expected and the response of each fungus to the compounds must be specifically studied. The results have also shown that the extracts of the bean could stimulate or inhibit both contaminant and non-contaminant species. A. niger tended to react to the compounds and extracts like the non-contaminant S. rolfsii.

The observation by Laycock (1931) that 76 per cent of fungal contamination of the bean cotyledon occurred through the micropylar end and the rest through other points was confirmed in this study. Other paths suggested are the raphe by Maravalhas (1966), and cracks in the testa as beans swell during fermentation. An experiment was conducted to find out the stage in cocoa bean curing when invading hyphae could be observed in the cotyledons. This would show whether invasion is associated with specific chemical changes in the bean.

It was observed that in the unfermented bean, 4 per cent of the beans contained hyphae in the micropylar region while another 4 per cent showed hyphae in the stylar region (see Table 14). The fungi isolated were Botryodiplodia theobromae and Fusarium decemcellulare. Spectrophotometric analyses had shown that the unfermented cotyledon was high in anthocyanin (0.16mg/g) and flavonol content (4.0mg/g) while the testa had these in lower quantities, 0.07 and 0.41 mg/g, respectively (see Table 7).

Compounds of extracts of the unfermented bean eluted from thin layer chromatographic plates had inhibitory effects on spore germination. Spot $N(C)_1$ depressed spore germination in A. flavus, A. fumigatus, A. niger, and A. tamarii and sclerotia of S. rolfsii (see Table 13A). It is indeed surprising that despite the presence of these compounds, B. theobromae and F. decemcellulare could invade the beans. It is possible that phenolic compounds alone do not confer resistance to fungal invasion in the beans. Seevers and Daly (1970) arrived at the same conclusion while working on wheat stem rust. They stated that the relationship between phenolic compounds and disease resistance had not been proven. Invasion, thereafter, went on steadily in the beans throughout fermentation and drying.

There was considerably higher infection, particularly by A. fumigatus and Mucor species of beans that had been fermented for 2 days with hyphae occurring in all parts of the bean except in the micropylar region.

On the last day of fermentation, that is, 6th day of curing, there was considerably higher fungal invasion of the beans. The micropylar and stylar regions showed greater infection than the mid-region and the principal invading fungi were Mucor pusillus, R. arrhizus and the Aspergillus species, A. fumigatus, A. restrictus and A. tamarii. The similarity of distribution pattern with the unfermented bean was striking, suggesting that invasion commences at both stylar and micropylar ends.

Finally, beans dried for 6 days after fermentation, that is, on the 12th day of curing revealed high contamination which was particularly severe at the micropylar and stylar ends. The major species being *Mucor* sp., *Penicillium* sp. and three Aspergilli, *A. chavalieri*, *A. flavus* and *A. tamarii* (see Table 14).

The occurrence of the phenolic compounds during these events did not portray any particular pattern, neither was the frequency of mycoflora occurrence associated with specific levels of phenolic compounds. Thus as shown in Table 7, free phenols remained almost at the same level on the 2nd and 6th days of fermentation while fungal invasion increased. Also, contamination was very high on the 6th day of curing when the content of orthodihydric phenols had risen tremendously from 0.33 mg/g to 2.18 mg/g on the 2nd day and then to 3.41 mg/g on the 6th day (see Tables 8 and 9).

Fungus species associated with contamination of cocoa beans included those with cellulolytic and those without known cellulolytic properties; eg. *Penicillium* species, *A. flavus*, *R. arrhizus* and *A. tamarii*. Fungi without known cellulolytic properties could have entered the cotyledons through paths created by those with cellulolytic properties. During entry, the fungi must have withstood the inhibitory effects of the phenol compounds, Pridham (1960) suggested that fungi employed two methods for detoxifying chemical barriers during the infection process. Such compounds were either glycosylated by the addition of sugar groups thus changing their chemical nature or the toxic compounds were solubilized so that compounds insoluble at physiological pH values, would then be dissolved to allow for their translocation.

Some of the factors that could predispose cocoa beans to fungal infection have been studied. The disorganisation of the sclerotised layer of bean testa during fermentation (Roelofsen, 1958), and the breakdown of cellular structure around the dome of the micropylar region (Dade, 1929) were not investigated in this study. These earlier reports could be confirmed in future studies.

Since some of the phenolic compounds have been found to inhibit fungal spore germination, further studies should identify potent compounds for possible future use in the control of cocoa bean fungal contaminants. Another recommendation that could be made is that fermentation time should be shortened to four days, using smaller heaps and the drying time also reduced possibly through the use of artificial drying methods in order to shorten the favourable period for invasion and thus minimise fungal contamination.

SUMMARY

1. The average annual fungal contamination of cocoa beans at Tema from October 1978 to July 1979 was 4.9 per cent.
2. The main crop cocoa beans had lower fungal contamination of 3.9 per cent and the minor crop beans a higher percentage of 6.4 per cent.
3. The average moisture content of cocoa beans at Tema warehouse was 6.4 per cent.
4. High incidence of fungal contamination was associated with high bean moisture content.
5. Twenty-one species of fungi belonging to nine genera were isolated from contaminated cocoa beans in addition to the presence of some sterile mycelia.
6. The most important fungal genera were *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* with the Aspergilli constituting the dominant flora, particularly those of the *Aspergillus glaucus* group; *A. chevalieri*, *A. ustus* and *A. ruber*.
7. *A. chevalieri* was the predominant contaminant of stored cocoa beans.
8. *Aspergillus phoenicis*, *Rhizopus oryzae*, and *Neurospora crassa* were being recorded on stored cocoa beans for the first time.
9. Testa extracts of unfermented beans supported vegetative growth in *A. flavus*, *A. fumigatus*, *A. niger*, *A. tamarii* and *Sclerotium rolfsii*.

All *Aspergillus* species grew better than *S. rolfsii* in the testa extracts of the unfermented beans, with *A. tamarii* producing the greatest amount of mycelium.

10. Extracts of testa of fermenting beans inhibited growth of the five fungi, *S. rolfsii* was the most severely inhibited fungus.

11. Extracts of testa of beans being dried after fermentation also depressed vegetative growth in the five species but to a lesser extent than extracts of fermenting beans.

12. In Czapek Dox broth at 25°C, *A. tamarii* grew best, producing mean mycelium dry weight of 287.2 mg and *A. nidulans* grew poorest producing 126.2 mg dry weight of mycelium.

13. In this medium, *A. fumigatus* grew fastest, reaching maximum growth in 5 days of incubation and *A. nidulans* slowest, attaining maximum growth after 14 days of incubation.

14. In Sabouraud's broth at the same temperature, however, *A. fumigatus* grew best and *A. tamarii* the poorest, But on Sabourauds agar medium *A. nidulans* grew slowest as measured by colony diameter at both 25° and 30°C.

15. The unfermented cocoa bean cotyledons and testa have phenolic compounds in varying concentrations. The amounts of these substances changed during fermentation and drying.

16. The quantities recorded could be summarised as follows:

(a) <u>Free phenols in cotyledons</u>

Unfermented cocoa bean : 4.00 ± 1.11 mg/g
Bean fermented for 2 days : 1.13 ± 0.82 "
Bean fermented for 6 days : 0.73 ± 0.16 "
Bean dried for 6 days : 0.70 ± 0.20 "

(b) <u>Free phenols in testa</u>

Unfermented cocoa bean : 0.41 ± 0.09 mg/g
Bean fermented for 2 days : 0.45 ± 0.08 "
Bean fermented for 6 days : 0.32 ± 0.11 "
Bean dried for 6 days : 0.22 ± 0.06 "

(c) <u>Orthodihydric phenols in cotyledon</u>

Unfermented cocoa bean : 0.33 ± 0.18 mg/g
Bean fermented for 2 days : 2.18 ± 0.47 "
Bean fermented for 6 days : 3.41 ± 0.09 "
Bean dried for 6 days : 2.42 ± 0.88 "

(d) <u>Orthodihydric phenols in testa</u>

Unfermented cocoa bean : 2.43 ± 0.22 mg/g
Bean fermented for 2 days : 2.19 ± 0.72 "
Bean fermented for 6 days : 3.15 ± 0.81 "
Bean dried for 6 days : 2.46 ± 0.95 "

(e) Flavonols in cotyledons

Unfermented cocoa bean : 0.21 ± 0.25 mg/g

Bean fermented for 2 days : 0.39 ± 0.16 mg/g

Bean fermented for 6 days : 0.72 ± 0.09 "

Bean dried for 6 days : 0.49 ± 0.11 "

(f) Flavonols in testa

Unfermented cocoa bean : 0.51 ± 0.15 mg/g

Bean fermented for 2 days : 0.33 ± 0.14 "

Bean fermented for 6 days : 0.43 ± 0.10 "

Bean dried for 6 days : 0.37 ± 0.04 "

(g) Anthocyanin in cotyledon

Unfermented cocoa bean : 0.16 ± 0.02 mg/g

Bean fermented for 2 days : 0.13 ± 0.02 "

Bean fermented for 6 days : 0.20 ± 0.05 "

Bean dried for 6 days : 0.14 ± 0.01 "

(h) Anthocyanin in testa

Unfermented cocoa bean : 0.07 ± 0.02 mg/g

Bean fermented for 2 days : 0.07 ± 0.01 "

Bean fermented for 6 days : 0.15 ± 0.05 "
Bean dried for 6 days : 0.24 ± 0.05 "

17. Testa extracts of unfermented beans showed four chromatographic spots of phenolic compounds of Rf values of 0.35, 0.48, 0.60 and 0.70 when solvent n-butanol-acetic acid-water was used.

18. The number of chromatographic spots obtained from extracts of unhydrolysed testa of beans cured for 0, 2, 6 and 12 days was 4, 3, 1 and 0 respectively with the respective Rf values of 0.35, 0.48, 0.60 and 0.70 for unfermented beans and 0.35, 0.48 and 0.62 for beans in 2 days of fermentation. The spots for extract of beans in 6 days of fermentation had an Rf value of 0.69.

19. The number of chromatographic spots obtained from extracts of hydrolysed testa of beans cured for 0, 2, 6, and 12 days was 2, 1, 1 and 0 respectively with the respective Rf value of 0.35 and 0.57 for unfermented beans. The single chromatographic spots observed for extracts of bean cured for 2 and 6 days was 0.57.

20. The percentage of conidia of _A. flavus. A. fumigatus. A. niger_ and _A. tamarii_ and sclerotia of _S. rolfsii_ which germinated in PDA "flood water" at 25°C after 50 hours was 27, 71, 33, 30 and 41 per cent respectively.

21. The germinating sclerotia of _S. rolfsii_ produced many hyphae. Among the Aspergilli, _A. fumigatus_ had the longest germ tubes, a mean of 7,110 um, and _A. tamarii_ the shortest - a mean of 1,950 um.

22. Compounds eluted from chromatogram spots had varying effects on the conidia of the Aspergilli and sclerotia of _S. rolfsii_

23. Compound from spot $N(0)_1$ of Rf 0.42 of the extract of non-hydrolysed testa of unfermented beans inhibited germination of conidia of all the Aspergilli and sclerotia of *S. rolfsii*, and reduced growth of germ tubes and hyphae.

24. Compounds eluted from the spot $N(2)_1$ of 0.31 of the extract of unhydrolysed testa from beans fermented for 2 days also inhibited germination of the conidia of all the Aspergilli and sclerotia of *S. rolfsii*. It, however, depressed germ tube growth in *A. fumigatus* and *A. tamarii* while it stimulated that of *A. flavus* *A. niger* and *S. rolfsii*.

25. Compound eluted from chromatographic spot $H(2)_1$ of Rf value 0.23 of extract of hydrolysed testa of beans on second day of fermentation severely depressed germ tube growth in *A. flavus*, *A. fumigatus* and *A. tamarii* but improved germ tube growth in *A. niger* and *S. rolfsii*.

26. Compound eluted from chromatographic spot $H(2)_2$ of Rf 0.54, of extract of hydrolysed testa of beans fermented for 2 days reduced conidial germination in *A. flavus*, *A. fumigatus* and *A. tamarii* but stimulated it in *A. niger* and improved germination of *S. rolfsii* sclerotia.

27. Compound from chromatographic spot $H(6)_1$ of Rf 0.23 of extract of hydrolysed testa of beans fermented for 6 days also depressed spore germination in *A. flavus*, *A. fumigatus* and *A. tamarii* but

stimulated germination in A. niger and S. rolfsii. It however, enhanced germ tube growth in all the species except A. tamarii

28. Unfermented cocoa beans, beans being fermented and those being dried after fermentation all had fungal contaminants, but infection was greater in beans being fermented or being dried.

29. Aspergillus and Mucor species were the major flora.

30. The fungi were not evenly distributed in the infected cotyledon. They occurred in greater quantities in the micropylar and stylar regions of the bean than in the mid-regions.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to my supervisor Dr. C.E. Amoako-Nuama who guided me during the course of these investigations and for her constructive criticism and suggestions during the preparation of the manuscript.

I am very grateful to Professor G.C. Clerk, for his initial help with this study before he left for Sabbatical Leave and for his general interest and advice.

My thanks and deep appreciation go to the Director of the Cocoa Research Institute, Tafo, and especially Dr. Daniel Adomako, Mr. O-A. Danquah, Mr. P. Akuley and Mr. Bossman, of the Physiology Department of the Institute for their help with the analyses of phenolic compounds. I also wish to record my sincere thanks to Mr. K. Asiakwam for his help with statistical analyses and Mr. E. K. Anyomi who typed the thesis.

I wish to acknowledge with thanks, the major financial support of the Ghana Cocoa Marketing Board, and a study leave, which enabled me to carry out this work.

Finally, my sincere grateful thanks go to my wife, Apini, for her patience, encouragement and support during the course of this work; to my niece, Ayama, for her dedication; and to my son, Abu for his refreshing innocence; and for bearing my long absence from home.

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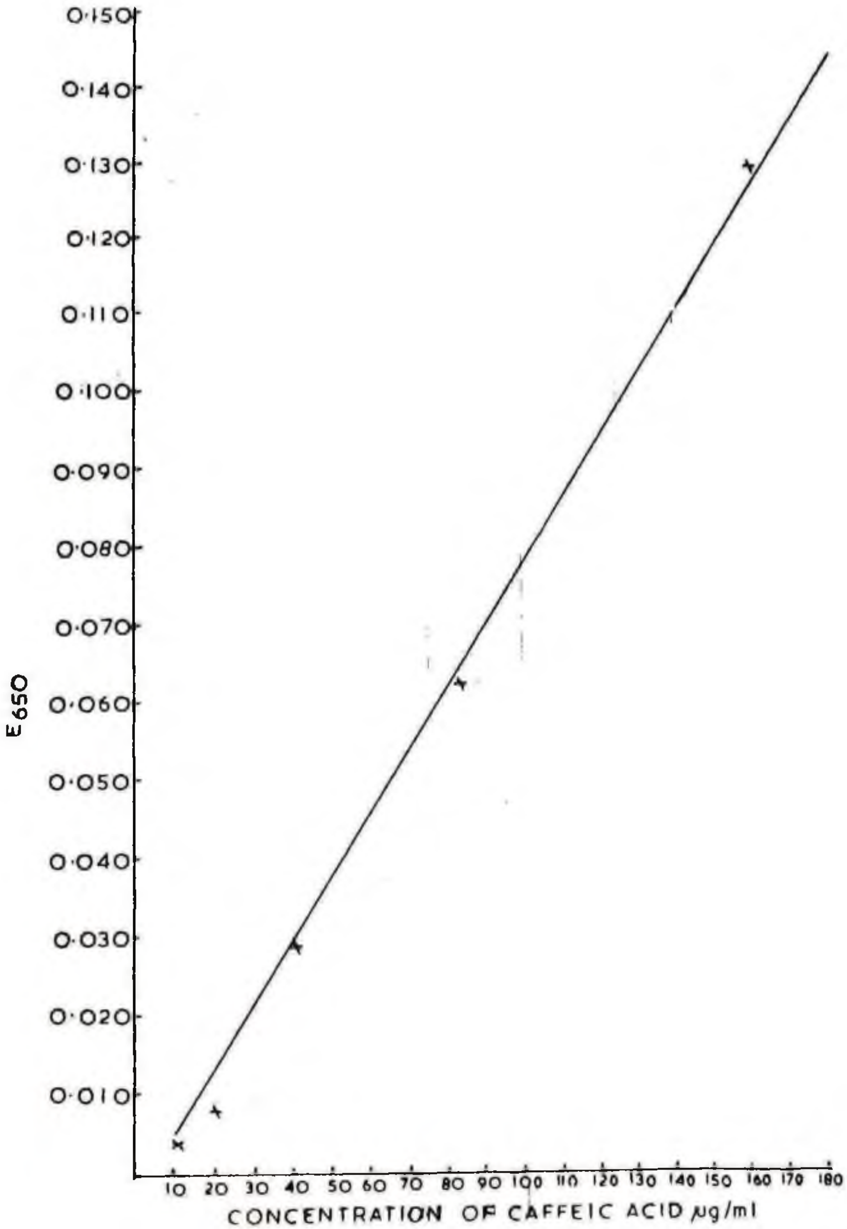
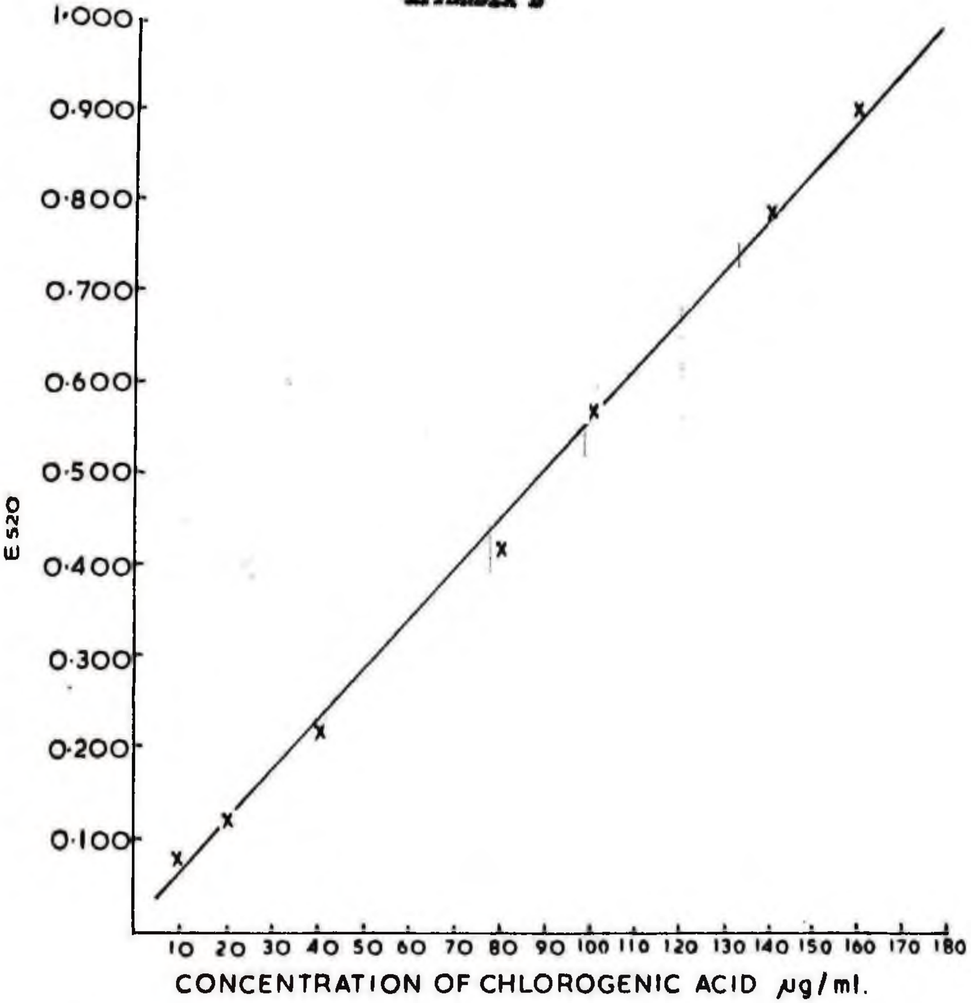
APPENDIX A



STANDARD CALIBRATION CURVE FOR FREE PHENOLS

APPENDIX B



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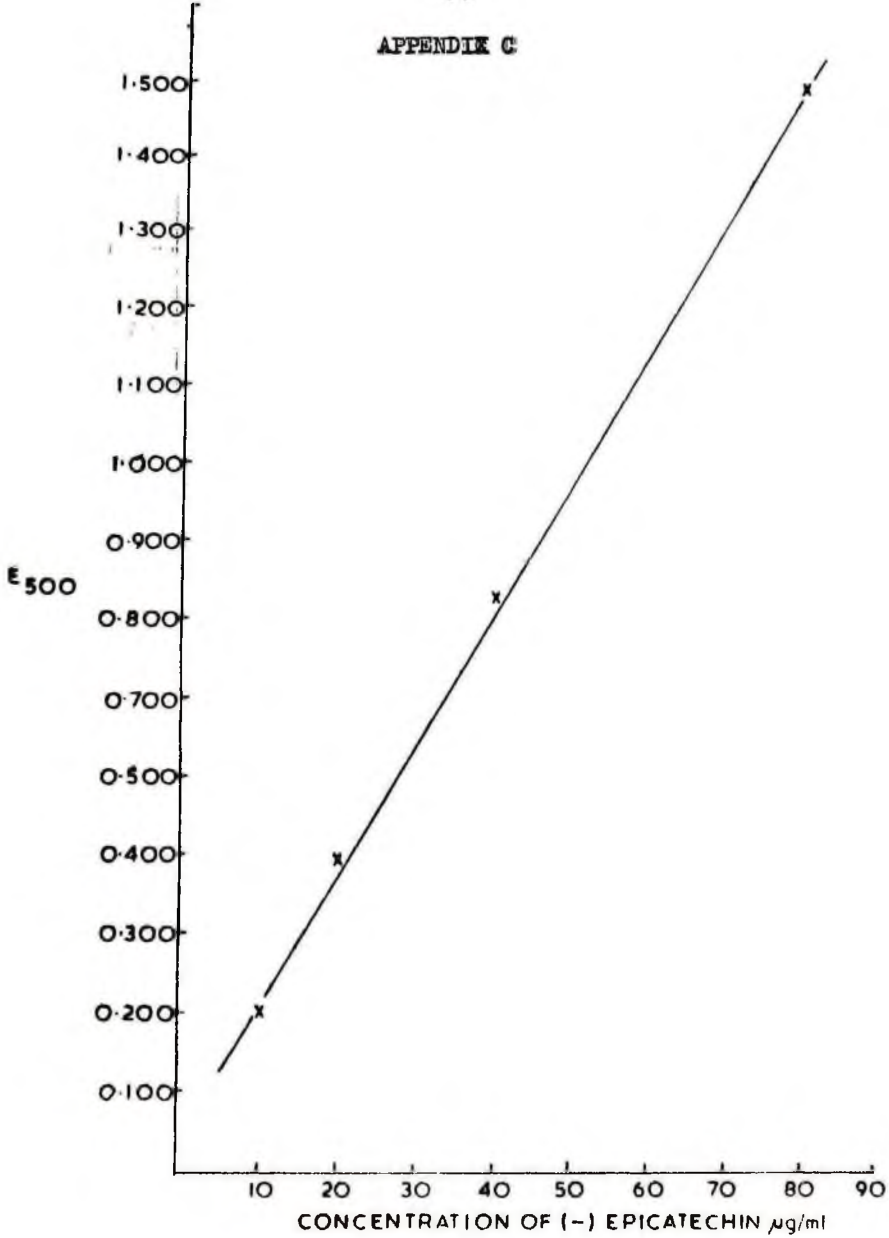
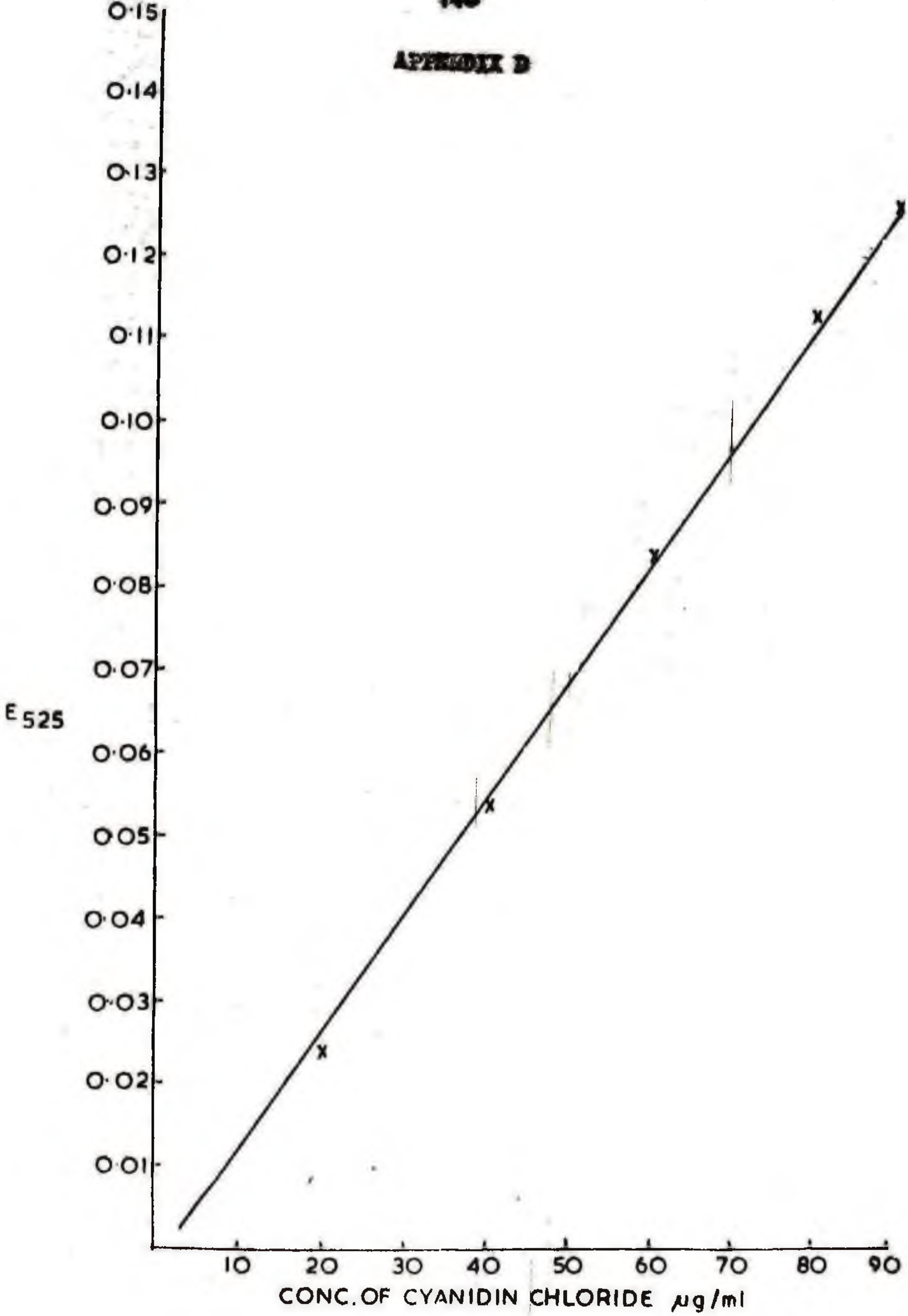
APPENDIX C



STANDARD CALIBRATION CURVE FOR FLAVONOLS

APPENDIX D



STANDARD CALIBRATION CURVE FOR ANTHOCYNIN