INVESTIGATION OF THE PHYTOALEXIN RESPONSE OF ARACHIS HYPOGAEA L. AND ITS POSSIBLE INVOLVEMENT IN RESISTANCE TO FOLIAR PATHOGENS

by

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Dedicated to my parents
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Investigation of the phytoalexin response of *Arachis hypogaea* L. and its possible involvement in resistance to foliar pathogens.

Natural infection of groundnut foliage with *Cercospora arachidicola* and *Phoma arachidicola* resulted in the accumulation of at least six antifungal compounds. The dominant antifungal compound was the pterocarpan medicarpin.

Abiotic agents including ultra-violet irradiation, salts of heavy metals and detergents were used to elicit medicarpin and other antifungal compounds. Abiotic elicitors induced low concentrations of compounds compared with those in infected material; for example leaves treated with silver nitrate accumulated medicarpin in the range 0-25 μg/g fresh weight whereas concentrations in infected leaves ranged from 150-800 μg/g fresh weight.

The more abundant antifungal compounds in extracts from infected leaves were isolated and purified by a combination of flash chromatography and semi-preparative high performance liquid chromatography (HPLC).

Purified compounds were identified by their UV absorption, mass spectrometry and proton nuclear magnetic resonance spectroscopy with confirmation by on-line spectroscopic techniques including; liquid chromatography with diode array detection, gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS).

Besides medicarpin other major phytoalexins were the isoflavone formononetin, the pterocarpan demethylmedicarpin and an isoflavanone. In addition minor phytoalexins were tentatively identified as 7-8'-dihydroxy-4'methoxy isoflavanone, an isoflavanone with unknown substitution and the isoflavone daidzein. An antifungal α β unsaturated aldehyde, occasionally found in leaves was identified as trans-trans 2-4 decadienal.

Greenhouse experiments with two cultivars originating in Zimbabwe, P84/5/244 and Egret, infected with *C. arachidicola* and *Puccinia*
arachidis, causal agents of early leafspot and rust respectively, resulted in differential accumulation of phytoalexins. In all experiments cv. P84/5/244 exhibited greater susceptibility and produced only small amounts of phytoalexins. On the other hand cv. Egret appeared to be more resistant and displayed a corresponding increase in phytoalexin accumulation.

Field data demonstrated that accumulation of phytoalexins reflected plant genotype and was not associated with resistance of some cultivars to *P. arachidicola*.

Analysis of the composition of phytoalexins indicated that demethylmedicarpin could be a metabolite of medicarpin. This was supported by demonstration of the ability of *Cercospora arachidicola* to degrade medicarpin *in vitro*. *Phoma arachidicola* also modified and degraded medicarpin but the major metabolites were not identified.
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ABBREVIATIONS

CH₃CN - acetonitrile (methyl cyanide)
cv - cultivar
EI - electron impact
EtOAc - ethyl acetate
EtOH - ethanol
GC-MS - gas chromatography mass spectrometry
g.f.wt. - gram fresh weight
HPLC - high performance liquid chromatography
i.d. - internal diameter
LC-MS - liquid chromatography mass spectrometry
m/z - mass to charge ratio
PDA - potato dextrose agar
POA - peanut oatmeal agar
S.D. - sample standard deviation
SPE - solid phase extraction
TIC - total ion chromatogram
TLC - thin layer chromatography
TMS - trimethylsilyl
\( t_R \) - retention time
UV - ultra violet
Chapter 1

INTRODUCTION

The plant

The cultivated groundnut (*Arachis hypogaea* L.) originates in South America and belongs to the subfamily Papilionaceae of the Leguminoseae.

The plant is an erect ascending or trailing herbaceous annual 15-70 cm in height. Leaves are evenly pinnate with two opposite pairs of leaflets. They are oblong ovate and about 4-8 cm long. The flower is yellow, papilionate and borne above ground on the vines. On fertilization the flower wilts and after a period of 5-7 days a positively geotropic peg (ovary) emerges and grows downwards into the soil. There it penetrates to a depth of 2-7 cm where pods form and ripen. The pod is a single loculed dehiscent legume which splits along a longitudinal ventral suture. The number of seeds produced per pod can vary from one to six. Groundnuts are grown in the semi-arid tropics between 40° north and 40° south of the equator in the semi-arid tropics (SAT). The plants require loose structured, fertile soil with good drainage.

The groundnut kernels are extremely nutritious containing 25-30% protein, 40-50% fat as well as cysteine, thiamine, riboflavin and niacin (St. Angelo and Mann, 1973).

Economic importance

The groundnut is one of the world's major crops and in 1983 it was estimated that 18.92 million hectares were planted, yielding a harvest of 19.9 million tonnes, with an average yield of dried pods of 1056 kg/ha (FAO Trade Statistics, 1984). Asia is the largest producer followed by Africa, North and Central America and South America. Approximately 80% of world production comes from developing countries where average yields are around 800-900 kg/ha compared with 2500 kg/ha in developed countries.

Two thirds of the world's production are used in the production of
a high quality oil which is made by boiling crushed nuts in vats or extraction under hydraulic pressure of 2-3 tonnes. This may be used for cooking or processed further into products such as margarine or soap. High quality oil is used in the pharmaceutical industry. The meal left after extraction is usually used as an animal feed supplement.

In developing countries the best crops are sold and the poorer ones used for food. In such areas the whole plant is important since the foliage can be used for animal feed and the shells for boiler fuel, fillers for fertilizers and in the manufacture of bricks.

Disease is one of the most important yield constraints, especially in the developing countries. Major diseases are caused by fungi and viruses. *Sclerotium minor, S. sclerotiorum* and *Calonectria crotalariae* are fungi responsible for causing severe pod and root rots (Porter, 1980; Beute, 1980). Other pod rotting fungi include *Fusarium* sp., *Macrophomina phaseolina* and *Rhizoctinia solani*.

A major post-harvest disease is caused by *Aspergillus flavus*, a fungus which produces highly toxic secondary metabolites known as aflatoxins which affect human health (Heathcote and Hibbert, 1978a and b).

Virus diseases are a serious problem in the SAT and include tomato spotted wilt virus (TSWV) and peanut mottle virus (PMV: Porter et al., 1982). However, fungal foliar diseases are responsible for greatest yield losses on a worldwide basis (Gibbons, 1979; Smith and Littrell, 1980).

Major diseases of groundnut

*Early leafspot*

This disease is prevalent in all groundnut growing countries although incidence and severity varies from one growing season to another.
Symptoms

Early leafspot is first recognizable as a small necrotic fleck which enlarges from 1-10 mm in diameter and becomes darker in colour and often is surrounded by a yellow halo (plate 1). Sporulation usually occurs on the adaxial surface of the leaf although conidia are often absent. Lesions can develop on petioles, stipules, stems and pegs in the later stages of an epidemic (Garren and Jackson, 1973).

Causal organism

Early leafspot is caused by *Cercospora arachidicola* Hori. (imperfect stage). The perfect stage of the fungus (*Mycosphaerella arachidis* Deighton) has been described in the United States but there is no convincing evidence that ascospores are an important initial source of inoculum (Smith and Littrell, 1980). Fruiting is amphigenous although conidia are formed primarily on the upper surface. Stromata are dark brown (25-100 μm in diameter) and conidiophores form dense fascicles of five to many. They are darker at the base, mostly once-geniculate, unbranched and septate. Subhyaline conidia are olivaceous, obclavate and mildly curved with truncate base and a subacute tip; they are divided by up to 12 septa. (Porter *et al.*, 1982; McDonald *et al.*, 1985).

Disease cycle

Conidia produced on crop residues are the main source of initial inoculum. They germinate forming one to several germ tubes, which enter open stomata or penetrate directly through epidermal cells. Leaf cells are killed in advance of proliferating intercellular hyphae. During long periods of wetness and temperatures from 25 to 31°C, lesions develop within 10-14 days. Conidia are dispersed by wind, splashing water and insects.

Control

Partial control may be achieved with good crop management practices such as crop rotation (Kucharek, 1975), removal or burial of crop residue (Garren and Jackson, 1973) and planting when conditions are unfavourable for disease development.
Early leaf spot is commonly controlled with multiple applications of fungicidal sprays of such compounds as benomyl, captofol, chlorothalonil, mancozeb and maneb (Smith and Littrell, 1980). These are generally applied by tractor-propelled sprayers, aircraft, sprinkler irrigation systems and controlled droplet application equipment.

**Late leaf Spot**

This disease is very similar to early leafspot, and although they may be found together it is usual to find that one predominates.

**Symptoms**

As with early leaf spot the disease is first recognizable as small necrotic flecks which enlarge to dark brown lesions of between 1-10 mm diameter on the abaxial surface of the leaf. It is unusual to find lesions of late leaf spot surrounded by a yellow halo. However this is not regarded as a reliable means for distinguishing it from early leafspot. Conidia are normally visible by eye in late leaf spot lesions.

**Causal organism**

As with early leaf spot the perfect state of the pathogen (*Mycosphaerella berkeleyii* W.A.Jenkins) is rarely found and it is the imperfect state, *Cercosporidium personatum* (Berk.and Curt.) Deighton, which is commonly found on lesions. It fruits on both sides of the leaf but it is more common on the lower surface. Dense, pseudoparenchymatous stromata are up to 130 μm in diameter, pale to olivaceous brown, smooth, geniculate and continuous or sparingly septate. Conidiophores commonly form dense fasicles in concentric rings. The presence of a thickened band on one side of the conidiophore distinguishes the fungus from members of the genus *Cercospora*. Conidia are pale brown, cylindric, obclavate, straight or slightly curved with a finely roughened wall that is rounded at the apex. They are normally 3-4 septate (McDonald et al., 1985).
Disease cycle

This is very similar to that of early leaf spot except that Cercosporidium personatum produces intercellular botryose haustoria.

Control

C. personatum is normally controlled by the methods described for C. arachidicola although there have been advances in biological control using the mycoparasite, Dicyma pulvinata (Mitchell and Taber, 1986).

Rust

Groundnut rust is caused by Puccinia arachidis Speg.. Before 1969 it was confined to South and Central America but it now may be found in most groundnut growing areas of the world. Alone or in combination with another disease such as late leaf spot, rust can result in devastation (Hammons, 1977; Porter et al., 1982; Subrahmanyam et al., 1985; Savery et al., 1988).

In the Caribbean, rust is such a problem that groundnuts cannot be grown on a commercial scale. Yield losses from rust alone of 50 and 70% have been reported in Texas and similar losses have been reported in India.

Symptoms

Like many rusts, the fungus is recognised by rust coloured pustules, otherwise known as uredia, predominantly on the abaxial surface of the leaf (plate 1). In heavily infected plants they may be found on the adaxial surfaces and on the stems. The uredia rupture to release reddish brown uredospores which are dispersed by wind and insects. In contrast to other major leafspots, severely infected leaves remain attached to the plant.

Causal organism

Puccinia arachidis Speg., the causal organism, is a rust with a simple life cycle in contrast to others such as P. graminis. The uredinial stage is that most commonly observed although teliospores have been detected on wild Arachis sp.. Spermogonia, aecia,
metabasidia and basidiospores are not known (Hennen et al., 1987). Uredia are predominantly located on the underside of the leaf, scattered or irregularly grouped; round, ellipsoid or oblong and dark cinnamon brown when mature. Uredospores are broadly ellipsoid or obovoid, brown walled and finely echinulate. They usually have two germ pores which are almost equatorial, often in flattened areas.

Telia, also mainly found on the abaxial surface of leaves, are chestnut or cinnamon brown, becoming grey or black after germination. Teliospores are oblong, obovate, ellipsoidal, or ovate, with a rounded to acute thickened apex. They are predominantly two celled.

**Disease cycle**

Uredospores represent the main means of dissemination of the fungus. However, they are short lived in crop debris and the fungus is therefore unlikely to survive if the break between crops is more than four weeks. The practice of continuous cultivation in many countries such as China and India seems to be an important factor in disease perpetuation. Volunteer plants may also be important but, as yet, no alternative hosts outside the genus *Arachis* are known.

Optimum conditions for infection include temperatures between 20-30°C, availability of water on the leaf surface and relatively high humidity.

**Control**

Good crop management can control the disease to a certain extent as mentioned above.

Sprays of Bordeaux mixture and dithiocarbamate were quite effective in controlling rust as are chlorothalonil and calixin (Subrahmanyam and McDonald, 1983).

Breeding for rust resistant cultivars has been a major undertaking at ICRISAT Centre, Patancheru, India, where a number of genotypes of cultivated groundnut and wild *Arachis* spp. which exhibited some resistance have been used in breeding programs (Subrahmanyam et al., 1983).
Web Blotch

Web blotch, caused by the fungus *Phoma arachidicola* Marasas, Pauer and Boerema, was first observed in Texas during the 1972 growing season (Pettit *et al.*, 1973) but it had been reported earlier in Russia, Brazil, Argentina and Zimbabwe. On a worldwide basis, web blotch is of minor importance compared to other leafspot pathogens. The appearance of this pathogen diminished following an increase in planting of Florunner, a runner-type cultivar with moderate resistance to web blotch and a decrease in the planting of highly susceptible Spanish-type cultivars.

**Symptoms**

Web blotch is characterized by two distinct symptoms in the field (plate 1). A webbing or netlike pattern of tan or bronze colour may appear on the adaxial surface of the leaves. Alternatively it may be apparent as larger almost circular tan to dark brown blotches which may coalesce to cover the whole leaf surface. Lesions on the abaxial surface are generally less pronounced and pale brown. Severe defoliation may occur, especially if other leaf spot fungi are also present.

**Causal organism**

As already mentioned the causal organism is the fungus *P. arachidicola* and a sexual stage may also observed in infested crop residues. This has been the subject of taxonomic confusion but is now designated *Didymella arachidicola* (Choch.). Taber, Pettit and Philley (Taber *et al.*, 1984).

Cultures grown *in vitro* on malt agar at 25°C are typically grey/olive in colour with a loose felty appearance at the periphery. The mycelium is 2.0-8.0 μm in diameter, olivaceous to brown and septate. It forms dark chlamydospores both aerially and submerged. When incubated at 18°C under near-ultraviolet light, brown pycnidia form within 5-7 days. Pycnidiospores are formed in basipetal succession on short conidiogenous cells and, under high humidity, are exuded through the ostiole as droplets. Although one cell they may
become septate as they mature.

Pseudothecia are dark brown, globose, short beaked or unbeaked, and 60-147 μm in diameter. Asci are bitunicate and cylindric to cylindric-clavate. Ascospores are one-septate, smooth and hyaline to pale yellowish, darkening with age.

*Disease cycle*

In the field pycnidiospores and ascospores serve as inoculum. Germ tubes penetrate directly through the cuticle resulting in a swelling. Networks of individual hyphae ramify under the cuticle and kill adjacent cells resulting in the webbed appearance. Hyphae also penetrate to subepidermal tissues. Proliferation of hyphae and subsequent extensive cell necrosis produce the typical blotch symptom. The disease is more severe during cooler seasons or at slightly higher altitudes where temperatures are cooler. Although the groundnut is the only known natural host, experimental infections have shown the legumes sweet clover and hairy vetch to be susceptible.

*Control*

Fungicides such as chlorothalonil and mancozeb plus benomyl have been shown to provide some control although, as already mentioned the runner cultivars which have some resistance are commonly planted.
Plate I - Important foliar diseases of groundnut (A) early leafspot caused by *Cercospora arachidicola* (top), (B) rust caused by *Puccinia arachidis* (centre) and (C) web blotch caused by *Phoma arachidicola* (bottom).
PLANT DEFENCE MECHANISMS

Only a small proportion of microorganisms are able to colonize living plants and these have acquired the ability to overcome the plant's numerous defence mechanisms. First line defences include protective structural barriers such as the cuticle and cell wall which prevent most microorganisms from penetrating. Pathogens can overcome these by gaining entry via natural openings such as stomata and lenticels, by direct mechanical penetration or by production of specific degradation enzymes (Kolattukudy and Köller, 1983; Cooper, 1983). The mechanisms involved are highly specific as demonstrated by rust fungi where specific leaf surface topography is essential for initiation and development of infection structures (Hoch et al., 1987).

Once a pathogen has penetrated the cuticle and cell wall, its progress may be restricted by post-infectional structural alterations including lignification, suberization and production of hydroxyproline rich glycoproteins (HRGPs). In some systems constitutive low molecular weight antifungal compounds have been implicated in resistance. The most studied plant defence mechanism is that of the inducible low molecular weight compounds, the phytoalexins which are antibiotic. These mechanisms are discussed briefly to gain an overview of plant defence reactions.

PRE-EXISTING STRUCTURAL DEFENCES

The cuticle

In aerial organs of plants this is the first barrier and consists of the insoluble polymer cutin which is embedded in hydrophobic materials known as wax. Depolymerization studies with analysis by GC-MS have shown that cutin is a polyester composed of \( C_{16} \) and \( C_{18} \) families of hydroxy and epoxy fatty acids (Walton and Kolattukudy, 1972).

Primary cell walls

Cell walls are comprised of polysaccharides and glycoproteins. In many situations cell walls may be strengthened by overlaying or
Impregnation with materials such as cutin, suberin, waxes, lignin, silicon and calcium.

Suberized cell walls

Suberin is a polymeric material associated with waxes similar to those associated with cutin (Kolattukudy, 1981). Ultrastructural studies have shown that suberized layers are deposited on the cell wall just outside the plasma membrane. During this process the cellular contents degenerate, leaving only cytoplasmic remnants in the lumen of highly suberized cells (Kolattukudy, 1981).

Lignin

Lignin is an aromatic phenolic polymer which is resistant to degradation by most microorganisms (Kirk, 1971; Kirk et al., 1979). It is formed by oxidative polymerization of three hydroxycinnamyl alcohols; trans-p-coumaryl, trans-conferyl and trans-sinapyl (Gross, 1978, 1979; Halbrock and Grisebach, 1979). The proportions of monomer units is variable, thus it is not possible to give an accurate description of the polymer. Lignin is deposited between cellulose microfibrils thus providing rigidity and resistance to compressive forces and degradation. Although preformed lignin is not a major defence mechanism, there are cases where the presence of preformed lignin has been associated with restriction of fungal growth such as in Puccinia graminis f.sp. tritici colonization of wheat stems where the fungus was unable to colonize adjacent lignified sclerenchyma (Hursh, 1924).

Silicon

Deposits of hydrated silica (SiO₂.nH₂O) were associated with resistance of rice to blast disease caused by Pyricularia oryzae. Enhanced deposition of silica and resistance to the pathogen were observed when soil was supplemented with silicate (Akai and Fukutomi, 1980). Silica has also been shown to accumulate as a reponse to infection of mesophyll cell walls in bean leaves with cowpea rust using electron probe microanalysis (Heath, 1979). However, if healthy bean tissues were treated with bean rust exudates or infected with
the fungus no silica was detected. Subsequent treatment of the same leaves with the cowpea rust did not induce accumulation of silicon. This research suggested that the success of the pathogen was dependent, in part, on the suppression of silicon accumulation. This may be seen as an example of non-host resistance.

Calcium

Increases in calcium and associated cross linking to pectic components in the cell wall was correlated with an observed increase in resistance of tissue to degradation by fungal polygalacturonases (Bateman and Lumsden, 1965). Successful pathogens have been shown to overcome the structural influences of calcium by producing oxalic acid which complexes the calcium allowing enzymic hydrolysis of pectic fraction to occur (Bateman and Beer, 1965).

POST INFECTIONAL STRUCTURAL DEFENCES

Induced structural alterations are an important resistance mechanism in plants. These responses include alterations or additions to existing cell walls by lignification and other strengthening materials as already described, formation of other barriers and vascular occlusion.

Induced lignification

Lignification occurs in response to infection or attempted infection by filamentous fungi, bacteria and some viruses (Vance et al., 1980). It has also been induced in wheat leaves by chitin (Pearce and Ride, 1982) although the response is not induced by the range of biotic and abiotic elicitors demonstrated to be effective in phytoalexin elicitation.

The composition of the lignins produced in such responses differ from those occurring naturally in healthy tissues but composition also varies between species.

Lignification appears in general to be a mechanism of non-host resistance. This was demonstrated when wounded wheat leaves
responded to non-pathogenic fungi with the accumulation of lignin around the wound in advance of the hyphae (Ride, 1975). Similar experiments using pathogens such as *Fusarium graminearum* and *Penicillium* species showed a much slower response (Ride, 1983). However some cases of race-specific resistance have been recorded where accumulation of lignin was associated with a hypersensitive response (Beardmore *et al.*, 1983; Hammerschmidt *et al.*, 1984). Lignification has also been implicated in the systemic resistance of cucumber to *Colletotrichum lagenarium* and *Cladosporium cucumerinum* (Hammerschmidt and Kuč, 1982).

There are several explanations of the involvement of lignin in plant defence as proposed by Ride (1978):

(a) The polymer may make host walls more resistant to mechanical penetration.

(b) Lignification may render host cell walls more resistant to fungal degradation by shielding the polysaccharides or by chemically altering them. This has been supported by the fact that lignified tissues in wheat leaves were resistant to enzyme attack (Ride, 1975, 1980). It has been shown more recently that indeed lignin-carbohydrate complexes are important in increasing resistance to breakdown and subsequent nutrient release in ruminants. Such complexes may have a role in plant defence (Wallace *et al.*, 1991).

(c) The impermeable nature of the lignin may limit diffusion of enzymes and toxins from fungi and conversely nutrients and water from the host to the pathogen.

(d) Low molecular weight phenolic precursors and free radicals produced during polymerization may be toxic to the fungus or inhibit essential enzymes.

(e) It is possible that the hyphal tips may become lignified based on the demonstration that chitin, cellulose and HRGP (all occurring in fungal cell walls) can serve as matrices for *in vitro* polymerization of lignin precursors.

*Accumulation of hydroxyproline-rich glycoproteins (HRGPs)*

The production of HRGPs is a well-documented response of many plants to infection by a wide variety of microorganisms (Clark *et al.*,...
1981; Esquerré-Tugayé et al., 1979; Hammerschmidt et al., 1984; Leach et al., 1982). Work with the melon system showed that the HRGPs accumulated faster and to higher concentrations in cultivars resistant to *Colletotrichum lagenarium* (Esquerré-Tugayé et al., 1979). More recently, using immunocytochemical localization of the HRGPs, a rapid accumulation was observed after tomato root cells were inoculated with a compatible pathogen. The response appeared to be initiated after contact between the pathogen and the plant cell wall, occurring as a result of tissue damage (Benhamou et al., 1990).

The glycoproteins have a linear structure which could act as a wall strengthening component. They are also agglutinins, since they contain a large number of basic amino acids and may act as polycations, aggregating negatively charged particles (Leach et al., 1982). The HRGPs in different dicotyledons have been shown to be similar by cross reaction of tomato HRGPs with antibodies raised against melon HRGPs (Benhamou et al., 1990). This observation supported earlier work where HRGPs from melon and tomato were found to have similar amino acid residues, sugars and molecular weight (Mazau et al., 1988; Smith et al., 1984).

The production of HRGPs as a plant defence mechanism bears similarities to phytoalexin accumulation including; (1) HRGPs accumulate more rapidly in incompatible reactions. (2) There are some cases of race-cultivar specific interactions (Hammerschmidt et al., 1984). (3) The response may be elicited by molecules of host or pathogen origin (Roby et al., 1985). (4) mRNAs increase earlier in incompatible interactions (Showalter et al., 1985).

**Deposition of new wall-like material**

Another common response of the plant to infection is the deposition of new wall-like material on the internal or external surface of the cell wall. Deposits on the interior wall are known as papillae. Attempted penetration of plants, by fungi, results in the formation of a papilla between the plasmalemma and the cell wall of the host at a point opposite the site of attempted penetration.

Papillae have been shown, by histochemical stains, to contain callose, a β 1,3-glucan, lignin, polysaccharides and cellulose (Ride...
and Pearce, 1979).

There have been many correlations between the production of papillae and resistance to fungal penetration (Aist, 1976). One study showed that successful infections only occurred when papillae were absent or poorly developed in reed canary grass infected with pathogens and non-pathogens. If systems where papillae were formed were treated with cycloheximide the formation of the papillae was prevented and the fungus became established (Vance and Sherwood, 1976).

Studies with wheat leaves infected by non-pathogens demonstrated the production of lignified papillae (Ride and Pearce, 1979). Lignified papillae were resistant to cell wall degrading enzymes, but chemical delignification rendered them vulnerable to the enzymes (Ride, 1978; Ride and Pearce, 1979). Other components of papillae may include suberin, protein, silicon, calcium, cellulose and phenolics depending on the host and pathogen involved (Ride, 1983).

Renewed cell division

Another common response of plants to wounding or infection is the formation of a zone of 'barrier' cells which separate healthy from infected cells, often resulting in desiccation thus preventing the pathogen from establishing necessary nutrient relationship. These new cells often contain suberin, lignin and other unidentified strengthening components. In wounded oaks deposition of suberin appeared to be crucial to resistance of the barrier zone (Pearce and Rutherford, 1981).

Vascular occlusion

This is an important mechanism of defence against fungi which penetrate the vascular system e.g. *Fusarium oxysporum* and *Verticillium albo-atrum*. These fungi spread through plants primarily as spores which are carried up in the transpiration stream. Spores are naturally trapped at vessel ends in the perforated plates, but successful pathogens are able to grow through them or produce secondary spores. Vascular occlusion occurs above these natural sites and involves the formation of tyloses and gel or gum plugs (Beckman
Rapid formation of tyloses and gels have been associated with resistance to vascular pathogens and slow or poor formation has been associated with susceptibility (Beckman and Talboys, 1981).

**ANTIMICROBIAL COMPOUNDS**

**Constitutive low molecular weight antimicrobial compounds**

Some of the first studies on why some plants exhibit resistance were those involving low molecular weight compounds. There is great diversity in the chemical nature of the compounds identified and in most cases they have been shown to be involved in non-host or race non-specific resistance. Some compounds inhibit germination of spores, such as the water soluble phenolics protocatechuic acid and catechol. These compounds occur in the outer pigmented scales of onion bulbs. Resistance to smudge disease caused by *Colletotrichum circinans* was shown to be a result of these phenolics diffusing from the dead scales into the inoculum droplets where they inhibited spore germination (Link *et al*, 1929; Link and Walker, 1933).

**Cyanogenic glycosides**

This group of compounds represent a major group of antifungal compounds and has been demonstrated in 800 plant species belonging to 70 families (Eyjolfsson, 1970). Although they are considered constitutive, it is as a result of damage that activity occurs and toxic HCN is released. The concentrations of HCN released at infection sites during fungal attack are sufficiently high to kill or inhibit growth of invading hyphae although there are no reports of the actual concentrations in the immediate vicinity of the fungi (Myers and Fry, 1978).

Studies with these compounds have indicated a role in non-host resistance. This was further supported by the finding that pathogens of cyanogenic plants were tolerant of HCN and this was due to the production of an enzyme, formamide hydro-lyase which catalyzes the
conversion of HCN to less harmful formamide. Analysis of other pathogens and non-pathogens of cyanogenic plants showed that all the pathogens were capable of producing high or moderate levels of this enzyme (Fry and Evans, 1977). Although there was a good correlation between tolerance of HCN, and presence of the enzyme, providing circumstantial evidence for the involvement of cyanogenic glycosides in non-host resistance it is apparent that the pathogens examined were not virulent on other cyanogenic hosts implying other virulence and resistance factors are involved.

Saponins

The saponins form the largest group of preformed inhibitors and they can be divided into steroid-like types of triterpenes, spirostanols, furostanols and alkaloids. Their mode of action is similar in that they destroy the selective permeability of cell membranes. This is due to complex binding of the saponins to membrane sterols resulting in loss of membrane elasticity, followed by leakage of cell constituents and organelle breakdown. Saponins are presumed to be located in vacuoles of intact cells at concentrations that vary depending on the part of the plant and the stage of growth.

One interesting study involving tomato-Fusarium solani showed the saponin tomatine to be important in resistance of green fruits to this fungus, which attacked all other parts of the plant. The fungus was inhibited by concentrations of 100 µg/ml⁻¹ in vitro. Mutants were obtained which were able to grow on media containing tomatine concentrations of 800 µg/ml⁻¹. The mutants were able to rot the green fruits owing to the low sterol content of the fungal cell membrane. Subsequent genetic analysis demonstrated that virulence to green fruits was correlated with low sterol content of fungal membranes and insensitivity to tomatine (Defago and Kern, 1983; Defago et al., 1983).

Other studies which have supported a role for saponins in disease resistance are those on tuliposides in resistance of tulip bulbs to Fusarium oxysporum f. sp. tulipae and avenacins in resistance of oats
to the fungus *Gaummannomyces graminis* (Mansfield, 1983; Schönbeck and Grunwaldt-Stöcker, 1986; Crombie *et al.*, 1986).
INDUCIBLE LOW MOLECULAR WEIGHT COMPOUNDS

Phytoalexins

Phytoalexins were first described as "antibiotics which are the result of an interaction of two different systems, the host and the parasite and which inhibit the growth of the microorganisms pathogenic to plants" (Müller and Bürger, 1940). After forty years of extensive studies on the accumulation of phytoalexins they were redefined as "low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plant cells after exposure to microorganisms" (Paxton, 1981). Some phytoalexins occur constitutively, such as the pterocarpan medicarpin in the heartwood of *Pericopsis angolensis* and *Swartzia madagascarensis* (tropical members of the Leguminosae). Their presence in the heartwood was thought to contribute to termite resistance (Harper et al., 1969). However, phytoalexins are normally absent from healthy tissues.

Phytoalexins have been isolated from plants of many families including the Leguminosae, Solanaceae, Convolvulaceae, Malvaceae, Vitaceae, Orchidaceae and Gramineae. Phytoalexins from different plant families are chemically diverse but are usually similar within members of the family such as isoflavonoids from the Leguminosae and terpenoids from the Solanaceae. Although one phytoalexin may predominate in a particular species most plants produce several closely related phytoalexins. However, it has been demonstrated that in several systems the chemical nature of the phytoalexins produced depends on the part of the plant infected e.g. pigeonpea seeds synthesize four isoflavones and the isoflavanone, cajanol (Dahiya et al., 1984), whereas leaves accumulate a chalcone and two isomeric stilbenes (Cooksey et al., 1982).

Role of phytoalexins in disease resistance

Phytoalexins accumulate in response to infection by fungi, bacteria, viruses and nematodes. Their involvement in resistance is usually associated with interactions in which resistance is expressed following penetration and is associated with necrosis of plant cells.
in non-host resistance, race-non-specific resistance and race-specific resistance.

Extensive biochemical, genetic and ultrastructural studies on many host-pathogen systems over the last ten years have substantiated early theories that \textit{de novo} phytoalexin production may be responsible for resistance of some plants to both pathogens and non-pathogens (Dixon, 1986; Darvill and Albersheim, 1984; VanEtten \textit{et al}, 1989).

One of the best studied systems is that of soybean and the Oomycete pathogen \textit{Phytophthora megasperma} f.sp. \textit{glycinea} (\textit{Pmg}) which is the causal agent of root and stem rot. Following infection with an incompatible race of \textit{Pmg}, the levels of phytoalexins increased from undetectable amounts to >10% of the dry weight of the infected tissue within 24-28 hours after inoculation (Keen and Horsch, 1972). To determine more precisely if the phytoalexins were responsible for the inhibition of fungal growth during expression of resistance Hahn \textit{et al} (1985) developed a radioimmunoassay specific for the major soybean phytoalexin, the pterocarpan, glyceollin I. This was used in combination with an immunofluorescent stain for the fungal hyphae. Detailed examination of zoospore infected roots using these techniques showed that the timing of differential accumulation and the spatial distribution of glyceollin correlated with restriction of hyphal growth.

Other studies provided complementary evidence to support a role for phytoalexins in the observed resistance responses of soybean to \textit{Pmg}. These studies involved the \textit{in situ} inhibition of the enzyme phenylalanine ammonia-lyase (PAL) using L-2-aminoxy-3-phenylpropanoic acid. This is a crucial enzyme in the phenylpropanoid pathway, an essential part of glyceollin biosynthesis. Inhibition of PAL resulted in a normally incompatible reaction becoming compatible (Moesta and Grisebach, 1982).

Metabolic inhibitors such as actinomycin D and blasticidin S were used to demonstrate the requirement for the synthesis of new proteins for phytoalexin production since inhibition of protein synthesis resulted in incompatible reactions becoming compatible (Yoshikawa, 1978). Similar studies have shown that phytoalexin production is an
important and early defence response in potato infected by incompatible races of Phytophthora infestans and bean infected by Botrytis cinerea (Sato et al., 1971; Mansfield, 1980).

Elicitation of the phytoalexin response

Phytoalexin responses have been induced by many stimuli other than infection, illustrating the nonspecific nature of this response. These have included biotic compounds of microbial and plant origin such as polysaccharides, glycoproteins, peptides enzymes and fatty acids (Dixon, 1985). Abiotic elicitors such as salts of heavy metals and irradiation with UV light may also elicit a phytoalexin response (Bailey, 1982).

HIGH MOLECULAR WEIGHT COMPOUNDS

Protein, Lectins and Agglutinins

The role of lectins, which occur widely in plants, in plant defence has been investigated in several systems (Callow, 1977; Etzler, 1985). Lectins act as agglutinins by binding to structures such as cell surfaces through specific carbohydrate containing receptor sites. A lectin isolated from wheat embryos (wheat germ agglutinin) was shown to bind to Carbohydrates containing N-acetylglucosamine, a major component of chitin found in the cell walls of many fungi. This lectin bound to growing hyphal tips of Trichoderma viride and prevented further growth (Mirelman et al., 1975). However, more information on in vivo activity of lectins is required.

Proteins that inhibit cell wall degrading enzymes have been detected in a number of plants such as pea (Hoffman and Turner, 1982) and French bean (Cervone et al., 1987).

Enzymes

The production of enzymes such as 1,3-β-D-glucanase and chitinase may be induced upon infection by fungi.
Proteinase Inhibitors

Proteinase inhibitors may be induced by fungal and endogenous elicitor molecules (Esquerré-Tugayé et al., 1984; 1985; Ryan et al., 1985; Ryan, 1990). A chemically diverse group, proteinase inhibitors are often located, or may accumulate in storage organs of the plant where they protect against rotting organisms.
THE PROJECT

Foliar diseases are a major constraint on groundnut production in developing countries where growers are unable to afford fungicides and the facilities for application. Another problem is the development of fungicide tolerant pathogens (Clark et al., 1974).

Some success has been achieved in identifying groundnut genotypes with resistance to one or more of the foliar pathogens which have been utilized in breeding programs (Subrahmanyam et al., 1980a, b, 1982). The breeding of plants with durable resistance is the most practicable form of plant disease control in the third world. Traditionally, sources of resistance have been sought in centres of diversity of the plant and its wild relatives. Resistance genes from these sources have then been bred into agronomically acceptable cultivars. Unfortunately, this resistance does not always prove to be durable. A complimentary approach is to define the mechanism of resistance of the plant and incorporate these characteristics in suitable commercial cultivars. Alternatively, some method of enhancing the resistance mechanisms might be a suitable method of control.

Initial observations by Cole (1981) indicated that when groundnut foliage was limiting and infected by *C. arachidicola* there was little or no infection by *P. arachidicola*. When the canopy was not limiting the two pathogens spread independently. Extracts from leaves infected by *C. arachidicola* were inhibitory to *P. arachidicola*. The main antifungal component was later shown to be the pterocarpan medicarpin which was also detected in leaves infected by *P. arachidicola* (Strange et al., 1985). Quantitation of medicarpin in different cultivars infected by both pathogens revealed significant differences in levels accumulated (Strange et al., 1985).

These observations and results were the basis for examining the role of phytoalexins during infection of groundnut leaves with foliar pathogens.
Chapter 2
Abiotic Elicitation of Phytoalexins

INTRODUCTION

The pterocarpan, medicarpin was identified as the predominant antifungal component in groundnut foliage infected with C. arachidicola or P. arachidicola (Strange et al., 1985). Other antifungal compounds were detected by using TLC bioassays with the dark spored fungi Cladosporium cucumerinum and P. arachidicola but insufficient material was available for purification and subsequent identification. In order to examine the possible role of phytoalexins in resistance of groundnut foliage to leafspot pathogens it was essential to obtain milligram quantities of these other antifungal components for identification and subsequent toxicity studies.

Many chemicals have been used to elicit phytoalexins, including salts of heavy metals (e.g. mercury, copper and silver), respiratory inhibitors (e.g. sodium iodoacetate, potassium cyanide and 2,4 dinitrophenol) and surfactants (e.g. Triton X-100, Nonidet P40 and sodium dodecyl sulphate) as reviewed by Bailey (Bailey, 1982).

Phytoalexins may also be induced as a result of mechanical injury of plant tissues. Examples include; (a) elicitation of stilbenes in groundnut kernels (Aguamah, 1981; Wotton and Strange, 1985; Cooksey et al., 1988) where groundnut kernels were simply surface sterilized, sliced and incubated at 25°C for 24-48 h. (b) accumulation of phaseollin following localised freezing of French bean tissues (Rahe and Arnold, 1975).

Phytoalexins can be elicited by irradiation of plant tissue with short wave UV light (254 – 270 nm). The mechanism of elicitation was associated with the formation of pyrimidine dimers in the plant DNA (Reilly and Klarman, 1980; Beggs et al., 1985). Other abiotic agents have also been shown to cause conformational changes in the plant DNA with subsequent phytoalexin production (Hadowiger and Schwochau, 1971a,b; Langcake and Pryce, 1977).
Copper sulphate was used to elicit small quantities of medicarpin in groundnut leaves 50-80 µg/g.f.wt. (Strange et al., 1985; Ingham and Markham, 1982). The initial aim of the project was to find an elicitor capable of inducing concentrations of phytoalexins similar to those found in infected groundnut leaves. Thus avoiding the need for preparation and application of a suitable inoculum and maintaining large numbers of plants.

Another advantage of using elicitors is that a clear picture of the plant's phytoalexins and possible route of biosynthesis may be obtained whereas if a pathogen were present there would be a risk of phytoalexin metabolism providing an inaccurate idea of the plant's ability to synthesize phytoalexins.

Once a suitable elicitor had been obtained the aim was to investigate the potential of using the elicitor-plant phytoalexin response as an indication of the plant's true ability to produce phytoalexins and determine whether or not this represents the situation where there is resistance to pathogens in both greenhouse and field. This could ultimately lead to the development of an in vitro test to eliminate more susceptible genotypes.
MATERIALS AND METHODS

Chemicals

HPLC-grade hexane and ethyl acetate were obtained from Fisons (Loughborough, UK). Ethyl acetate and ethanol (GPR) were purchased from BDH (Poole, UK). Chemicals used as abiotic agents were of analytical grade from BDH or Sigma (Poole, UK).

Standard medicarpin was previously purified by semipreparative HPLC or synthesized by C.J. Cooksey (Chemistry Dept. UCL.).

Plant Material

Plants were grown in John Innes no.1 at 25-30°C. Detached leaves were used in all experiments, the youngest leaves were selected from plants which were between 6 and 12 weeks old.

The cultivars used were all of known genotype with the exception of the Brazilian cultivar Tatu which was used initially when there was no other germplasm. The repeated use of this cultivar was explained by the positive response to treatment. The other cultivars were obtained from ICRISAT and were either resistant or susceptible to one or more of the foliar pathogens. The cultivar Egret, grown commercially in Zimbabwe until recently, was also used in these experiments since earlier work showed that it accumulated large amounts of medicarpin and other antifungal compounds.

TREATMENT OF GROUNDNUT LEAVES WITH ABIOTIC ELICITORS

Ultra-Violet Irradiation

UV irradiation was initially investigated since previous studies with groundnut tissue (kernels and leaves) revealed that short periods of irradiation followed by incubation led to the accumulation of several antifungal compounds (Aguamah, 1981). The conditions optimised by Aguamah were adapted for these experiments.

The abaxial surface of excised leaflets (samples of 1 g.f.wt) were irradiated with short wave u.v. light using a CC20 light box
(BDH, Poole, UK) where the energy at the cabinet floor was 300 μwatts/cm².

The leaflets were irradiated for 0, 10, 20, 40 and 80 min.

The irradiated leaflets were placed in 9 cm petri-dishes containing a moistened 9 cm filter paper and incubated at 27°C in the dark for 72 hours.

Leaves were extracted and analysed for medicarpin as described below.

Elicitation with Silver Nitrate

Silver nitrate was used to elicit glyceollins in soybean cotyledons (Epperlein, 1985) and medicarpin and related isoflavonoids in chickpea cotyledons (Cooksey unpublished). Elicitor solution may be applied to leaves by one of several methods; these included floating the leaves on the surface of the test solution for several days (Ingham and Markham, 1982) or applying droplets to the leaf surface and incubating them for several days before removing and extracting the droplet (Harborne and Ingham, 1978; Ingham, 1981). Although the latter method is more commonly used, it has a major disadvantage in that the amount of phytoalexin extracted from the droplet is often only a fraction of what is produced. For these experiments the elicitors were applied as droplets but the whole leaf was extracted to recover maximum yields of phytoalexins.

METHOD

Solutions of silver nitrate (concentrations ranging from 10⁻¹ M to 10⁻⁸ M) were applied to the abaxial surface of excised leaflets as 5 μl droplets (1 g/f.wt. leaves x 2). Ten droplets were distributed evenly across each leaflet with the intention of obtaining discrete areas of damaged cells surrounded by healthy cells. The leaflets were placed on plastic gauzes on moist filter papers in 9 cm petri-dishes and incubated as in previous experiments.

In separate experiments silver nitrate was applied to leaves of differing age i.e. older waxy ones and newly opened ones. Leaves were extracted and analysed as described below.
Treatment of leaflets with mercuric chloride

Solutions of mercuric chloride were applied to excised leaflets as described in the protocol above.

Treatment of leaflets with copper Sulphate

The protocol used by Ingham and Markham (1982) was followed to examine the effectiveness of copper sulphate as an elicitor of medicarpin in groundnut leaves.

Excised leaflets were floated both abaxial and adaxial surface downwards on copper sulphate solution (0.25 g/100 ml) with control leaflets floating on distilled water. The plates were incubated at 27°C for 5 days before extraction and analysis.

Sodium iodoacetate

This respiratory inhibitor was examined as a potential elicitor as it had been successful in eliciting glyceollins in soybean leaves (Ingham et al., 1981). Concentrations ranging from 5 x 10^{-6} M to 10^{-1} M were applied to leaflets as described for salts of heavy metals with distilled water as a control.

Phytalexin Extraction

Phytalexins were extracted by facilitated diffusion as described by Keen (Keen, 1978) and as used successfully in initial work with the infected leaves (Strange et al., 1985). Leaves were placed in aqueous ethanol (60%; 15 ml/gram fresh weight) and a vacuum applied repeatedly until the solvent had been forced into the intercellular spaces. This was evident by the darkened colour and change in density. Samples were allowed to stand for 24 h. with regular agitation.

The ethanol was removed in vacuo at 45°C and the remaining aqueous fraction was partitioned three times against equal volumes of ethyl acetate. The ethyl acetate fractions were combined and dried over anhydrous sodium sulphate overnight. The ethyl acetate was decanted and dried down by rotary evaporation. The residue was
taken up in ethyl acetate to give a concentration of 1 g.f.wt./ml.

Analytical high performance liquid chromatography (HPLC).

The apparatus consisted of an Altex pump (Beckman Instruments Inc., Berkeley, USA), a Pye-Unicam UV detector (Philips Analytical, Cambridge, U.K.) and a Tekman chart recorder (Tekman Electronics Ltd., Bicester, U.K.). A steel column 250 x 4.6 mm i.d. packed with silica (Partisil 5) was used with an isocratic mobile phase consisting of hexane:ethyl acetate (3:1, v/v) at a flow rate of 1.5 ml/min. Samples were introduced to the column via a 20 μl loop attached to an Altex valve. The eluent was monitored at 290 nm with an absorbance range set at 0.04 AUFS.

Identification

The presence of medicarpin in extracts of leaves treated with abiotic elicitors was determined by retention of the unknown compared to the retention time of authentic material.

Quantification

Medicarpin was quantified by external standardisation using a calibration curve obtained by injecting different concentrations of medicarpin and measuring the resulting peak area.
RESULTS

Identification of medicarpin

Medicarpin was identified in extracts of leaves treated with various abiotic agents by comparing the retention time of the unknown to that of authentic material (fig. 2.1). No other antifungal compounds were detected when extracts from treated leaves were examined by TLC bioassay as described in chapter 3 (data not shown).

UV Irradiation

Irradiation with short wave UV light resulted in the accumulation of small amounts of medicarpin (table 2.1). Leaves were observed to be damaged by visualisation of fluorescent areas when irradiated with long wave UV. There was no significant difference in the concentrations of medicarpin accumulating in the cultivars resistant to foliar pathogens, ICG 4747 and ICG 7885 and the susceptible cultivar ICG 221. Little or no medicarpin was detected in extracts from ICG 7897 and untreated controls.
TABLE 2.1

Medicarpin accumulation in groundnut leaves irradiated with UV light

<table>
<thead>
<tr>
<th>Exposure (min.)</th>
<th>Amount of medicarpin in cultivars; (µg/g. f.wt.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICG 4747</td>
</tr>
<tr>
<td>0</td>
<td>8.40</td>
</tr>
<tr>
<td>10</td>
<td>20.56</td>
</tr>
<tr>
<td>20</td>
<td>22.40</td>
</tr>
<tr>
<td>40</td>
<td>15.61</td>
</tr>
</tbody>
</table>

* Single 1 g samples were used due to limited foliage
Figure 2.1 - Identification of medicarpin by comparison of retention time of standard medicarpin (peak 1) (a) to a peak of similar retention time in an extract of groundnut leaves (cultivar Egret) treated with silver nitrate (b) when analysed by normal phase HPLC.
Treatment with silver nitrate

Silver nitrate was tested on cultivars Tatu, ICG 221 and Egret (Table 2.2) and they all responded with the production of low concentrations of medicarpin. No medicarpin was detected in control samples.

TABLE 2.2

Medicarpin accumulation in groundnut leaves treated with silver nitrate

<table>
<thead>
<tr>
<th>Conc. AgNO₃</th>
<th>Amount of medicarpin in cultivars (mean ± S.D., n = 2)(μg/g.f.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TATU</td>
</tr>
<tr>
<td>10⁻¹ M</td>
<td>0</td>
</tr>
<tr>
<td>5x10⁻² M</td>
<td>13.99 ± 2.34</td>
</tr>
<tr>
<td>10⁻² M</td>
<td>9.89 ± 5.62</td>
</tr>
<tr>
<td>5x10⁻³ M</td>
<td>5.10 ± 3.83</td>
</tr>
<tr>
<td>10⁻³ M</td>
<td>0</td>
</tr>
<tr>
<td>0 M</td>
<td>0</td>
</tr>
</tbody>
</table>

When silver nitrate was applied to both older thick leaves and young thin leaves low concentrations of medicarpin were elicited. Similar concentrations of medicarpin were detected in older and younger leaves when treated with silver nitrate solutions of 5 x 10⁻²M and 5 x 10⁻³M (table 2.3) However, when leaves were treated with a
solution of $10^{-2}$M silver nitrate, the younger leaves accumulated less medicarpin.

**TABLE 2.3**

Accumulation of medicarpin in leaves from cultivar Egret, of different age, treated with silver nitrate

<table>
<thead>
<tr>
<th>Conc. AgNO₃</th>
<th>Amount of medicarpin (µg/g, f.wt.) (mean ± S.D., n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>older thicker</td>
</tr>
<tr>
<td>0 M</td>
<td>0.29 ± 0.50</td>
</tr>
<tr>
<td>$5 \times 10^{-2}$ M</td>
<td>12.96 ± 0.15</td>
</tr>
<tr>
<td>$10^{-2}$ M</td>
<td>14.32 ± 1.17</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$ M</td>
<td>11.28 ± 3.40</td>
</tr>
</tbody>
</table>
Treatment of leaves with mercuric chloride

Little or no medicarpin was detected in cultivar Tatu treated with HgCl₂ of different concentrations but 5-50 µg/g.f.wt. was detected in cultivar ICG 221 (table 2.4).

TABLE 2.4

Accumulation of medicarpin in leaves treated with mercuric chloride

<table>
<thead>
<tr>
<th>Conc. HgCl₂</th>
<th>Amount of medicarpin (µg/g.f.wt.) (mean ± S.D., n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICG 221</td>
</tr>
<tr>
<td>10⁻¹ M</td>
<td>0</td>
</tr>
<tr>
<td>5x10⁻² M</td>
<td>42.24 ± 33.88</td>
</tr>
<tr>
<td>10⁻² M</td>
<td>30.66 ± 6.17</td>
</tr>
<tr>
<td>5x10⁻³ M</td>
<td>5.50 ± 1.72</td>
</tr>
<tr>
<td>10⁻³ M</td>
<td>2.62 ± 3.70</td>
</tr>
<tr>
<td>5x10⁻⁴ M</td>
<td>4.33 ± 1.52</td>
</tr>
<tr>
<td>0 M</td>
<td>0</td>
</tr>
</tbody>
</table>
Treatment with copper sulphate

Solutions of cupric sulphate were used successfully by Ingham to elicit phytoalexins in several legumes but on this occasion low concentrations of medicarpin were obtained and medicarpin was also detected in control leaves (table 2.5). Medicarpin in the controls may be explained by the presence of contaminants in the water which had not been sterilised.

TABLE 2.5
Accumulation of medicarpin in leaves treated with cupric sulphate (0.25 g/100 ml)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Amount of medicarpin (μg/g.f.wt.) (mean ± S.D., n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAXIAL</td>
<td>20.75 ± 5.00</td>
</tr>
<tr>
<td>ABAXIAL</td>
<td>19.75 ± 5.30</td>
</tr>
<tr>
<td>CONTROL(DW)</td>
<td>8.20 ± 2.55</td>
</tr>
</tbody>
</table>


Treatment with sodium iodoacetate

Low concentrations of medicarpin were obtained when leaves were treated with the respiratory inhibitor, sodium iodoacetate (Table 2.6). Maximum medicarpin was detected in leaves treated with $5 \times 10^{-3}$ molar sodium iodoacetate. At concentrations of elicitor above and below this, the concentrations of medicarpin detected were <6 µg/g.f.wt.

**TABLE 2.6**

Accumulation of medicarpin in leaves, from cultivar Egret, treated with sodium iodoacetate

<table>
<thead>
<tr>
<th>Conc.</th>
<th>Medicarpin (µg/g.f.wt.)</th>
<th>(mean ± S.D., n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$M</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-2}$M</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>$10^{-2}$M</td>
<td>3.61 ± 3.39</td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-3}$M</td>
<td>15.69 ± 6.61</td>
<td></td>
</tr>
<tr>
<td>$10^{-3}$M</td>
<td>5.75 ± 2.47</td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-4}$M</td>
<td>4.93 ± 1.75</td>
<td></td>
</tr>
<tr>
<td>0M</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

The elicitors used to treat groundnut leaflets resulted in the accumulation of low concentrations of medicarpin. For such a method to be practical for groundnuts it was necessary to induce concentrations of medicarpin in excess of 200 µg/g.f.wt. Although this was not achieved there may have untested chemicals which may have had the desired effect, as in the case of soybean leaves where sodium iodoacetate induced the accumulation of the highest levels of phytoalexin when tested with many other chemicals (Keen et al., 1981). However, the experiences of other workers eliciting phytoalexins in groundnut leaves has also resulted in levels of medicarpin much lower than can be detected in the same cultivar during fungal infection (Ingham, Personal communication).

In all experiments the treated leaves had areas of discrete necrosis adjacent to intact healthy cells, which according to the theory of endogenous elicitation, should be sufficient to initiate phytoalexin synthesis and accumulation (Bailey, 1982). This may be the explanation for the accumulation of low levels of medicarpin in treated leaves but an enhancement of this response may only be achieved with the specific pathogen interactions. This was the case in chickpea where low concentrations of phytoalexins were elicited by plant enzymes or wounding but higher concentrations accumulated during infection with *Ascochyta rabiei* (Kessman and Barz, 1986).

In view of these results it was not practical to continue the search for the ideal abiotic elicitor, an alternative strategy was required. One possibility was the use of chemicals of fungal origin which had been successful in other systems. However, preliminary experiments using chitosan and cell wall degrading enzymes, resulted in the accumulation of low concentrations of medicarpin (data not shown).

Since certain groundnut cultivars, including Egret, produced high concentrations of medicarpin and other antifungal compounds during
infection with leaf spot pathogens, the isolation of these compounds from infected leaves was the most realistic approach.
Chapter 3

Isolation and Identification of Antifungal Compounds from Infected Groundnut Foliage

INTRODUCTION

In order to evaluate the significance of antifungal compounds in the resistance of groundnut foliage to leafspot pathogens it was essential to characterize the major antifungal compounds produced during host-pathogen interactions. Abiotic elicitation had been unsuccessful therefore it was necessary to isolate milligram quantities of compounds from infected foliage from cultivars previously demonstrated to accumulate high levels of antifungal compounds including the pterocarpan medicarpin (Strange et al, 1985).

Techniques such as thin layer chromatography (TLC) and column chromatography (CC) have been widely used for the purification of legume phytoalexins. More recently semi-preparative HPLC has been shown to be of great value for obtaining milligram quantities of phytoalexins. Large scale preparative chromatography was used to purify large amounts of stilbene phytoalexins from groundnut kernels (Aguamah et al, 1981). However where sample size is a limiting factor this method is not practical since the method of detection, by refractometer, is insensitive.

Characterization of phytoalexins involves the use of diagnostic reagents and various physiochemical techniques including ultraviolet spectroscopy, mass spectrometry and proton nuclear magnetic resonance spectroscopy (Ingham, 1982).

This chapter includes methods for extraction and purification of phytoalexins from infected groundnut leaves by a combination of chromatographic techniques. Classical methods such as UV spectroscopy were used for identification but in combination with on-line chromatography-spectroscopy techniques.

The diode array detector was an alternative to a single wavelength detector for the detection of components separated by
high pressure liquid chromatography (HPLC). This enabled a range of wavelengths to be scanned giving rise to a three dimensional chromascan of wavelength vs time vs absorbance (Fig 3.1a). The UV spectra at any retention time may be selected and compared to those of known standards with similar retention times (Fig.3.1b) thus providing rapid identity.

This technique was complimented by gas and liquid chromatography interfaced to mass detectors. GC-MS has been used successfully for the identification of legume phytoalexins (Woodward, 1980, 1981).

Liquid chromatography mass spectrometry has yet to gain popularity but recent developments in interface/ionization technology have shown the potential of this technique. The application of this technique to phytoalexin research is addressed.
Figure 3.1
(a) Three dimensional profile of eluting analytes with wavelength from 189 nm to 390 nm. The main advantage of this facility was to determine an optimum wavelength for routine single wavelength detection and obtaining a more accurate idea of sample purity.

(b) Rapid confirmation of compound identity (medicarpin) by normalisation and superimposition of uv spectra selected from peaks with corresponding retention times in authentic material and extracts from infected groundnut foliage.
EXPERIMENTAL

Chemicals

HPLC grade acetonitrile, methanol, hexane and ethyl acetate were obtained from Fisons (Loughborough, UK). Chloroform and acetic acid (AR) were purchased from May and Baker (Ongar, UK). Ethyl acetate and ethanol (GPR) were obtained from BDH (Poole, UK). Pure water was obtained from an Elga (High Wycombe, UK) pure-water system.

All other chemicals were of analytical grade and purchased from BDH or Sigma (Poole, UK). Spherisorb ODS1 (10 μm) and Partisil 5 (5 μm) were obtained from Phase Separations (Deeside, UK). Silica gel 60 and TLC plates were obtained from BDH.

The phytoalexin standards demethylmedicarpin, medicarpin and 7, 2’-dihydroxy-4’-methoxyisoflavone were gifts from Professor P.M. Dewick (Department of Pharmacology, University of Nottingham, Nottingham, UK). Medicarpin, formononetin and daidzein were gifts from Professor W. Barz (Lehrstuhl für Biochemie der Pflanzen, Universität Münster, Münster, Germany).

Extraction of phytoalexins

Groundnut leaves from the cultivars Flamingo and Egret (125 g fresh weight), infected with C. arachidicola, were collected from experimental plots near Harare, Zimbabwe and air dried. They were vacuum infiltrated with 60% ethanol and agitated at intervals for 24 h. The ethanol was removed in vacuo at 40 °C and the remaining aqueous solution partitioned three times against ethyl acetate. The ethyl acetate fractions were pooled and dried over anhydrous sodium sulphate.

Thin layer chromatography (TLC)

Aliquots of the ethyl acetate extracts were run on TLC plates (silica gel 60 on an aluminium support; Merck No. 3554) in a tank saturated with a solvent system consisting of ethyl acetate-cyclohexane (1:1, v/v). Developed plates were examined by UV irradiation of both long and short wavelength. Since the plates contained a fluorescent indicator, which absorbed light at 254 nm and
fluoresced at the green end of the spectrum, components absorbing at 254 nm were detectable since the fluorescence was quenched and they appeared as dark spots against a bright background. Exposure to long wave UV indicated the presence of any fluorescing compounds since this was characteristic of some legume phytoalexins (Ingham, 1982b) and in particular stilbenes which had been isolated from groundnut kernels and leaves (Aguamah, 1981).

Spots were also detected by exposure to iodine vapour or by spraying with diazotized p-nitroaniline (Smith, 1958). Carbonyl groups were detected by reaction with p-phenylenediamine (2%) in cold trichloroacetic acid (Spendley et al., 1982).

Antifungal compounds were located by spraying with a spore suspension of Cladosporium cucumerinum (Homans and Fuchs, 1970). The spore suspension was made up in half strength Czapek-Dox medium at a density of 0.8 absorbance units at 620 nm and, after spraying, the plates were incubated at high humidity in the dark for 48 h.

Isolation and purification of phytoalexins

Phytoalexins extracted from large samples (125 g), were separated by a two stage process consisting of flash chromatography (Still et al., 1978) and semi-preparative HPLC.

Flash chromatography

The ethyl acetate solution resulting from liquid-liquid extraction was evaporated to dryness by rotary evaporation. The residue was dissolved in 10 ml chloroform. In order to find solvent systems giving suitable resolution, small aliquots of the chloroform extract, (20 μl), were spotted onto silica gel TLC plates and developed in a combination of solvent systems. Separation was assessed by visualisation under UV light, exposure to iodine vapour and antifungal activity. Once a solvent system had been established the chloroform extract was separated on a column of silica gel 60 (Merck 40-60 μm; 230-400 mesh; 150 x 20 mm i.d.). The column was initially conditioned with 100% cyclohexane, the sample (5 ml) was applied and the phytoalexins eluted in a stepwise gradient of ethyl acetate in
cyclohexane starting with 100% cyclohexane. At each step of the gradient (100 ml) the ethyl acetate content was increased by 10% (v/v) until 50% was reached. This was followed by elution with 100% ethyl acetate. Solvent flow rate was 2 ml/min. Initially 10 ml fractions were collected and those with similar components were pooled but it was more convenient to collect larger fractions (50 or 100 ml). Fractions containing phytoalexins were examined by analytical HPLC or by chromatographing them on TLC plates.

**Semi-Preparative HPLC**

Phytoalexins in the fractions from the silica column were isolated by semi-preparative HPLC. The apparatus was as described on page 29 with a semi-preparative column (250 mm X 10 mm i.d.) of Spherisorb ODS 1 (10 μm). Samples (250-500 μl) were introduced to the column via a 2,000 μl loop and a Valco valve and eluted in acetonitrile-water (1:1, v/v). Fractions corresponding to absorption peaks were collected manually. Purity was checked by TLC, using iodine vapour to visualize the spots, and by HPLC with diode array detection (see below).

Antifungal compound(s) eluted in 100% cyclohexane were efficiently separated by normal phase semi-preparative HPLC using a silica stationary phase (Partisil 5) and a mobile phase consisting of hexane:chloroform 1:1 (v/v). Eluting analytes were monitored at 280 nm.

**Analytical HPLC**

A Philips apparatus was used, consisting of two pumps (PU 4100), an automatic sampler (PU 4700) and a diode array detector (PU 4021) interfaced to a trivector data system. Compounds were separated on a column of Spherisorb ODS 1 (10 μm; 250 X 4.6 mm i.d.) protected by an Upchurch low volume guard column (Anachem, Luton) packed with Techoprep C18 (25-40 μm). The mobile phase was a gradient of acetonitrile in 1% acetic in which the acetonitrile concentration was increased from 35% to 40% over the first 12 min. and then to 75% over the next 18 min. A reequilibration time of 5 min. was allowed to elapse before the next sample was injected. The flow rate was 1.5
ml/ min and the eluent was monitored at 290 nm at an absorbance range of 0.04 AUFS. Compounds were identified on the bases of retention time and comparison of their UV spectra with those of authentic samples. They were quantified by comparison of peak areas with those of external standards. Purity of isolated compounds was examined by comparison of UV spectra through a peak to determine homogeneity i.e. spectra from the upslope, the apex and the downslope.

Identification of phytoalexins

Ultra Violet Spectroscopy - The UV spectra of purified compounds were obtained on a Varian - Cary spectrophotometer. Methanolic solutions were scanned from 230 nm to 340 nm.

Purified compounds were quantified by their absorbance ($\epsilon = A/C\lambda$ where $A =$ absorbance, $C =$ concentration in g moles/L and $\lambda =$ path length (cm, usually 1) using previously calculated molecular extinction coefficients: medicarpin (287 nm) - log $\epsilon$ 3.90 (Smith and Mclnnes, 1971), isoflavanones (277 nm) log $\epsilon$ 4.00, formononetin (250 nm) - log $\epsilon$ 4.44 (Dewick, 1975) and demethylmedicarpin (287 nm) log $\epsilon$ 3.93 (Dewick and Steele, 1982).

HPLC-UV spectrophotometry

Samples were run on the analytical HPLC and the retention times and the spectra obtained from the diode array detector compared with those of authentic compounds.

Mass Spectrometry

A selection of complimentary techniques was used; gas chromatography mass spectrometry (GC-MS) provided electron impact spectra (EI) at specific retention times. For some compounds EI spectra were obtained by direct probe insertion. Liquid chromatography mass spectrometry (LC-MS) provided chemical ionisation (CI) spectra at specific retention times.

Gas chromatography mass spectrometry (GC-MS)

Initially the purified compounds were derivatised to form trimethylsilylethers. They were separated on a crosslinked
methylsiline capillary column (25 m with 0.25 μm film) attached to a Philips gas chromatograph. Helium was used as the carrier gas with a column head pressure of 10 psi. The GC was interfaced to a VG7070 mass detector and the electron voltage used was 70 ev with a source temperature of 220°C. A mass range of 35-700 was scanned every three seconds. The GC program started at 50°C increasing to 220°C at 10°/min followed by a further increase to 250°C at 3°/min.

The initial work on GC-MS was followed up with a more detailed study using authentic standards, purified compounds and extracts from infected material. A Carlo Erba Strumentazione HPGC 5160 was fitted with a BP1 fused silica/quartz capillary column (50 m with 0.5 μm film:SGE). Helium was the carrier gas with a column head pressure of 130 KPa. Dried samples were derivatised in a mixture of trimethylchlorosilane (Sigma) and pyridine (1:1) and the components were separated using the following GC program: 50°C increasing to 250°C at 40°C/min. with a further increase to 300°C at 5°C/min. where it was maintained for a further 15 minutes. Ions were detected by a Hewlett Packard 5970 series selective mass detector using an electron energy of 70 ev.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Additional confirmation of molecular mass was obtained by running samples on a Vestec 201 thermospray mass spectrometer with positive ion discharge (1000v). Samples were separated on a Hichrom ODS2 (3 μM particle size) column (150 X 4.9 mm i.d.). Solvent A was 20% CH₃CN/0.1 M ammonium acetate buffer and solvent B was 80% CH₃CN/0.1 M ammonium acetate. The gradient profile used maintained B at 15% for one minute with an increase to 25% over 12 min. with a further increase to 90% over the following 18 min. Start conditions were control 144°C, tip heater 288°C, source block 326°C and vapour 343°C. The column was reequilibrated by decreasing %B to 15% over 4 min. where it was maintained for 3 min. Flow rate was 1 ml/min. Analytes were also monitored at 254 nm for confirmation of separation.

The samples analysed by LC-MS had previously been analysed by analytical HPLC with diode array detection and components in the
sample were identified by comparison with standards. Although the LC conditions were different from those used with the diode array it was possible to compare peaks in both analyses by calculating retention times relative to medicarpin (see Appendix 1).

**Proton Nuclear Magnetic Resonance (¹H NMR)**

Spectra were obtained on a Varian 400 MHz spectrophotometer operating in the fourier transform mode.
RESULTS

EXTRACTION

Antifungal compounds were successfully extracted in aqueous ethanol with subsequent partitioning into ethyl acetate and separated by normal phase TLC (fig. 3.2). Examination of the plates under short wave UV light revealed that the areas that were subsequently antifungal also absorbed short wave UV light. Irradiation with long wave UV light demonstrated that none of the components fluoresced strongly.

ISOLATION AND PURIFICATION

Flash chromatography

A suitable solvent system was developed by separating small aliquots of the crude extract on TLC plates with different proportions of ethyl acetate and cyclohexane. Figure 3.3 shows the changes in Rf. values with increase in percentage ethyl acetate in the developing solvent. It can be seen that solvent of composition 45% or 50% ethyl acetate can separate ten antifungal compounds but that compositions containing less ethyl acetate resulted in migration of fewer components which could be advantageous in a stepwise gradient using these solvents in flash chromatography.

Initially 10 ml fractions were collected and analysed qualitatively by HPLC with diode array detection. Fractions containing components of the same retention time and UV spectra were pooled and the extract examined for antifungal activity. Table 3.1 summarizes the information obtained from the diode array for the pooled fractions.
TABLE 3.1
Preliminary analysis of major compounds in five pooled flash fractions of decreasing hydrophobicity separated by HPLC.

<table>
<thead>
<tr>
<th>FRACTION*</th>
<th>No.CMPDS.</th>
<th>rt.(sec.)#</th>
<th>λmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1300</td>
<td>278</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>800</td>
<td>252,301</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>468</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>950</td>
<td>288 (med)</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>450</td>
<td>288</td>
</tr>
</tbody>
</table>

* combined fractions from flash chromatography
b number of UV absorbing components
# samples were run on a reverse phase column with a mobile phase consisting of acetonitrile in 1% acetic acid in which the acetonitrile concentration was increased from 35% to 40% over the first 12 min. and to 75% over the next 18 min.

The antifungal activity of these fractions was examined and compared with the crude extract by TLC bioassay (fig. 3.4). The components in the fractions, with medicarpin in fraction 5 acting as a reference for polarity, exhibited antifungal activity. Examination of the developed plate before bioassay had shown that these components absorbed short wave UVlight.

For more convenience 2×50 ml fractions were collected at each gradient step and aliquots were removed and examined qualitatively and quantitatively by analytical HPLC with diode array detection. The resulting fractions contained between one and six antifungal compounds (fig. 3.5) which could easily be separated and purified by isocratic semi-preparative HPLC.
Figure 3.2

TLC bioassay of ethyl acetate extracts of groundnut foliage infected by *C. arachidicola* (lanes A to E) and medicarpin (lanes F and G) which served as a marker. Components were separated on silica gel 60 TLC plates using a solvent system consisting ethyl acetate:cyclohexane (50:50). Infected extracts contained at least six antifungal components, most of which were more polar than medicarpin.
Figure 3.3

Diagrammatic representation of separation of antifungal components (represented by each line) with increasing amounts of ethyl acetate. As seen in fig. 2 50:50 ethyl acetate:cyclohexane was selected as a solvent system for TLC analysis of extracts but the progressive separation was adapted for flash chromatography.
Figure 3.4

TLC bioassay of pooled flash fractions i.e. those of similar retention time and UV spectra when separated by analytical HPLC. Components were separated on silica gel 60 TLC plates using a solvent system consisting of ethyl acetate:cyclohexane (50:50). Flash fractions (lanes C to G) contained one or two major antifungal components in contrast to the original extract (lane A). Lane C represents fraction 5 as detailed in table 1, lane D; fraction 4, Lane E fraction 1, Lane F fraction 2 and lane G fraction 3. Lane B was a sample collected during HPLC and is not important in this instance.
Figure 3.5

Distribution of phytoalexins in fractions from flash chromatography on silica gel. Two 50 ml fractions were collected for each concentration of ethyl acetate in the mobile phase apart from the final fraction which was 100 ml when the solvent was 100% ethyl acetate.

Others were unidentified apart from the hydrophobic compound found in 0% ethyl acetate, which was identified as trans, trans-2,4 decadienal.
IDENTIFICATION

The results of various physicochemical methods and reaction with diagnostic reagents are summarized for each compound.

Medicarpin

Although previously identified in earlier studies, medicarpin was reidentified in the current study.

Medicarpin had a Rf. of 0.71 on TLC and gave a pale orange spot when sprayed with diazotized p-nitroaniline.

The absorption maxima in methanol were 282 nm and 286.5 nm (fig. 3.6). For regular rapid identification analytical HPLC with diode array detection provided spectral information at a particular retention time. Fig. 3.7a shows spectra taken from two peaks, one from synthesized medicarpin and the other from a peak of similar retention time in an infected extract. The peaks were normalised at 285 nm and superimposed for comparison. Spectral similarity indicated the two compounds were similar. Updated software enabled quantification of spectral similarity (fig. 3.7b).

GC-MS of the trimethylsilylether gave m/z = 342 (M+) with prominent peaks at 327 (M+ - 15), 219 (M+ - 123), 206 (M+ - 136), 164 (M+ - 178), 148 (M+ - 194) as shown in fig. 3.8. This was supported by further comparison of standards and infected extracts by GC-MS as summarised in table 3.2.
TABLE 3.2

Comparison of retention time and major fragments produced by electron impact (EI) ionisation of TMS derivatives of authentic medicarpin and an extract of groundnut foliage infected with *C. arachidicola* as analysed by GC-MS.

<table>
<thead>
<tr>
<th>Major ions [m/z(%)</th>
<th>SAMPLE</th>
<th>AUTHENTIC</th>
<th>INFECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_R$ (min.)</td>
<td>23.30</td>
<td>23.43</td>
</tr>
<tr>
<td>342 (100%)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>341 (37.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>327 (8.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>325 (5.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>219 (6.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>207 (3.9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>206 (9.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>191 (4.8%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>165 (2.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>164 (11.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>161 (12.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>149 (3.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>148 (17.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a - Base peak on which percentage abundance was calculated.

These results are consistent with a compound of molecular mass 270 with one substituent hydroxyl group.

**LC-MS**

Compounds previously identified by LC-UV in extracts from *Cercospora* infected leaves were examined by LC-MS. Medicarpin had a retention time of 24.72 min. and gave an $M^+$ of 271 consistent with a mass of 270 (fig. 3.9).
Figure 3.6 - Absorbance spectrum of medicarpin in methanol from 310 nm to 240 nm showing characteristic absorption maxima where $\lambda = 282$ nm (shoulder) and $\lambda = 286.5$ nm.
Figure 3.7 - (a) UV spectra obtained from standard medicarpin and unknown of similar retention time superimposed and normalised at 285 nm. (b) as (a) but with updated software facilitating quantitative comparison of standard and unknown spectra. In (b) maximum absorption is 289 nm as opposed to 285 nm in (a).
Figure 3.8 - Electron impact (EI) mass spectrum of TMS ether derivative of medicarpin separated by gas chromatography.
Figure 3.9 - Identification of medicarpin in an extract of groundnut foliage infected with *C. arachidicola* by LC-MS. Components were separated by reverse phase HPLC as shown by the total ion chromatogram (TIC; top). A mass chromatogram at m/z 271 (the mass of protonated medicarpin) gave one peak at $t_m$ 24.72 min. (centre) and the mass spectrum at this retention time showed ions of m/z 271 only (bottom).
Demethylmedicarpin

This compound had an Rf. value of 0.54 on TLC and gave a bright orange derivative on reaction with diazotized p-nitroaniline indicative of a hydroxyl substituent at C-9 (Ingham and Markham, 1980).

The absorption spectrum was similar to that of medicarpin indicating similar conjugation. The absorption maxima in methanol were 282 nm and 286.5 nm (fig. 3.10). Similar spectra were for the authentic demethylmedicarpin and a compound of similar retention time separated from extracts of peanut leaves infected with C. arachidica (fig. 3.11).

GC-MS of the trimethylsilyl ether gave m/z = 400 (M+) with prominent peaks at 385 (M+ - 15), 219 (M+ - 181), 206 (M+ - 194) and 185 (M+ - 215) as shown in fig. 3.12. This was supported by further studies with authentic material and infected extracts as summarized in table 3.3.
TABLE 3.3
Comparison of retention time and major fragments produced by electron impact ionisation (EI) of TMS derivatives of authentic demethylmedicarpin and an extract from groundnut foliage infected with *C. arachidicola* as analysed by GC-MS.

<table>
<thead>
<tr>
<th>Major ions [m/z (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMPLE</td>
</tr>
<tr>
<td>t_m(min.)</td>
</tr>
<tr>
<td>400 (100%)*</td>
</tr>
<tr>
<td>399 (27.8%)</td>
</tr>
<tr>
<td>386 (2.9%)</td>
</tr>
<tr>
<td>385 (9.2%)</td>
</tr>
<tr>
<td>383 (5.2%)</td>
</tr>
<tr>
<td>357 (1.2%)</td>
</tr>
<tr>
<td>327 (2%)</td>
</tr>
<tr>
<td>219 (10.4%)</td>
</tr>
<tr>
<td>207 (7%)</td>
</tr>
<tr>
<td>206 (13.6%)</td>
</tr>
<tr>
<td>191 (4.4%)</td>
</tr>
<tr>
<td>185 (6.2%)</td>
</tr>
</tbody>
</table>

* - Base peak on which percentage abundance was calculated.

Electron impact probe analysis gave major fragments at m/z 257 (16.2%), 256 (100%), 255 (54.1%), 239 (8%), 147 (21.9%), 134 (20.3%), 129 (10%) and 123 (7%).

These results indicated that the unknown compound had a mass of 256 amu. with two hydroxyl ring substituents.

LC-MS

Demethylmedicarpin was identified in extracts from infected groundnut foliage by LC-MS. A peak at retention time 12.60 min. had a
prominent ion $m/z = 257$. This result was consistent with a mass of 256 (fig. 3.13).

**Proton NMR**

Absolute confirmation was obtained from proton NMR spectra which compared well with literature values (table 3.4).

**TABLE 3.4**

Comparison of Proton NMR data for demethylmedicarpin isolated in this study with those in the literature (Dewick and Steele, 1982).

<table>
<thead>
<tr>
<th>CHEMICAL SHIFTS (ppm)</th>
<th>Literature values</th>
<th>Purified material</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.39d</td>
<td>7.39d</td>
<td></td>
</tr>
<tr>
<td>7.08d</td>
<td>7.08d</td>
<td></td>
</tr>
<tr>
<td>6.56dd</td>
<td>6.55dd</td>
<td></td>
</tr>
<tr>
<td>6.42d</td>
<td>6.41d</td>
<td></td>
</tr>
<tr>
<td>6.40dd</td>
<td>6.39dd</td>
<td></td>
</tr>
<tr>
<td>6.37d</td>
<td>6.35d</td>
<td></td>
</tr>
<tr>
<td>5.49d</td>
<td>5.48d</td>
<td></td>
</tr>
<tr>
<td>4.23m</td>
<td>4.2m</td>
<td></td>
</tr>
<tr>
<td>3.62t</td>
<td>3.62t</td>
<td></td>
</tr>
<tr>
<td>3.49m</td>
<td>3.49m</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.10 - Methanolic UV spectrum of demethylmedicarpin scanned from 310 nm to 250 nm showing characteristic absorption maxima of simple pterocarpans at $\lambda = 282$ nm (shoulder) and $\lambda = 286.5$ nm.
Figure 3.11 - Identification of demethylmedicarpin by analytical HPLC with diode array detection where a spectrum selected from standard was compared with a spectrum from a peak of similar retention time in an extract from infected leaves.
Figure 3.12 - Electron impact mass spectrum of TMS ether derivative of demethylmedicarpin.
Figure 3.13 - Identification of demethylmedicarpin in extracts from infected groundnut leaves by LC-MS. Components were separated by reverse phase HPLC as shown by the total ion chromatogram (top). A mass chromatogram at m/z 257 gave a peak at t_m 12.60 min. (centre). The mass spectrum of this peak contained ions of m/z 257 (bottom).
Formononetin

This compound had an Rf. of 0.42 on TLC and gave a very pale orange/yellow derivative when sprayed with diazotized p-nitroaniline. The absorption spectrum in methanol showed maximum absorbance at 249 nm and 299 nm (fig. 3.14). Similar spectra were obtained for authentic formononetin and a compound of similar retention time in extracts of infected leaves (fig. 3.15).

GC-MS of the trimethylsilyl ether gave m/z = 340 (M⁺) with prominent peaks at 325 (M⁺ - 15), 208 (M⁺ - 132), 163 (M⁺ - 177) and 132 (M⁺ - 208) (fig. 3.16). This was further supported by additional studies with infected material and authentic standards as demonstrated in table 3.5.

TABLE 3.5

Comparison of retention time and fragment ions produced by electron impact ionisation of TMS derivatives of authentic formononetin and an extract from groundnut foliage infected with *C. arachidicola* as analysed by GC-MS.

<table>
<thead>
<tr>
<th>Major ions [ m/z (%) ]</th>
<th>STANDARD</th>
<th>INFECTED EXTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>tᵣ (min.)</td>
<td>28.93</td>
<td>29.28</td>
</tr>
<tr>
<td>340 (100)</td>
<td>340 (100)</td>
<td></td>
</tr>
<tr>
<td>339 (33.1)</td>
<td>339 (33.2)</td>
<td></td>
</tr>
<tr>
<td>325 (36.5)</td>
<td>325 (26.7)</td>
<td></td>
</tr>
<tr>
<td>282 (3.6)</td>
<td>282 (12.8)</td>
<td></td>
</tr>
<tr>
<td>281 (4.5)</td>
<td>281 (31.9)</td>
<td></td>
</tr>
<tr>
<td>208 (9.2)</td>
<td>208 (16.2)</td>
<td></td>
</tr>
<tr>
<td>207 (7.9)</td>
<td>207 (47.1)</td>
<td></td>
</tr>
<tr>
<td>132 (18.8)</td>
<td>132 (12.6)</td>
<td></td>
</tr>
</tbody>
</table>

* Base peak on which percentage abundance was calculated
Electron impact spectra were also obtained by direct probe insertion and the ions at m/z 268 (100%), 267 (21%), 253 (13%), 225 (4.9%), 132 (51.5%) and 117 (8.6%) were obtained.

LC-MS

An MH⁺ of 269 was obtained at a retention of 21.72 min (fig. 3.17) indicative of a compound of mass 268 amu.

Proton NMR

Absolute confirmation was provided by proton NMR spectrum, and table 3.6 compares data for isolated formononetin to that obtained for authentic material.

**TABLE 3.6**

Comparison of proton NMR data for purified formononetin and authentic material

<table>
<thead>
<tr>
<th>CHEMICAL SHIFTS (ppm.)</th>
<th>Standard formononetin*</th>
<th>Purified formononetin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.33s</td>
<td>8.17s</td>
<td></td>
</tr>
<tr>
<td>8.02d</td>
<td>8.05d</td>
<td></td>
</tr>
<tr>
<td>7.53d</td>
<td>7.54d</td>
<td></td>
</tr>
<tr>
<td>6.98d</td>
<td>6.98d</td>
<td></td>
</tr>
<tr>
<td>6.97dd</td>
<td>6.96dd</td>
<td></td>
</tr>
<tr>
<td>6.88d</td>
<td>6.90d</td>
<td></td>
</tr>
<tr>
<td>3.82s</td>
<td>3.83s</td>
<td></td>
</tr>
</tbody>
</table>

* Spectrum obtained from formononetin in DMSO

* Spectrum obtained in acetone- d₆
Figure 3.14 - Methanolic absorption spectrum of formononetin scanned from 320 nm to 240 nm. Characteristic absorption maxima were obtained at 249 nm and 299 nm.
Figure 3.15 - Identification of formononetin by comparison of UV spectra selected from standard and unknown peaks during reverse phase HPLC using updated software (Philips Chromascan 6003).
Figure 3.16 - Electron impact mass spectrum of TMS ether derivative of the isoflavone formononetin.
Figure 3.17 - Identification of formononetin in an extract of groundnut foliage infected with *C. arachidicola* by LC-MS. Components were separated by reverse phase HPLC as shown by the total ion chromatogram (top). An ion chromatogram at m/z 269 (centre) showing one major peak at 21.72 min. The mass spectrum of this peak (bottom) shows the presence of one major ion at m/z 269.
**Isoflavanone I**

This compound, tentatively identified as 7,4'-dimethoxy-2'-hydroxyisoflavanone, had an Rf. value of 0.46 on TLC and produced a pale yellow orange derivative when sprayed with diazotized p-nitroaniline. The absorption maxima in methanol were 277 nm and 311 nm (fig. 3.18). No authentic material was available for chromatography and spectral comparison.

GC-MS of the trimethyl silyl ether gave m/z = 372 (M⁺) with prominent peaks at 357 (M⁺ - 15), 209 (M⁺ - 163), 193 (M⁺ - 179), 164 (M⁺ - 208), 149 (M⁺ - 223) and 121 (M⁺ - 251) as shown in fig. 3.19.

Additional studies using GC-MS were consistent. Although no authentic material was available, purified material was used as a standard for comparison with crude extracts from infected leaves (Table 3.7), confirming this to be a major compound in the response of groundnut leaves to infection by leafspot pathogens.

**TABLE 3.7**

Comparison of retention times and fragment ions produced by electron impact ionisation of TMS derivatives of purified isoflavanone I and an extract from infected groundnut leaves as analysed by GC-MS.

<table>
<thead>
<tr>
<th>Major ions [ m/z (%) ]</th>
<th>STANDARD</th>
<th>INFECTED EXTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMPLE tᵣ (min.)</td>
<td>28.29</td>
<td>28.65</td>
</tr>
<tr>
<td>372 (19.2)</td>
<td>372 (19.2)</td>
<td></td>
</tr>
<tr>
<td>357 (3.7)</td>
<td>357 (2.6)</td>
<td></td>
</tr>
<tr>
<td>207 (10.4)</td>
<td>207 (27.4)</td>
<td></td>
</tr>
<tr>
<td>165 (13.5)</td>
<td>165 (10.8)</td>
<td></td>
</tr>
<tr>
<td>164 (100)*</td>
<td>164 (100)</td>
<td></td>
</tr>
<tr>
<td>149 (36.6)</td>
<td>149 (37.9)</td>
<td></td>
</tr>
<tr>
<td>121 (20.3)</td>
<td>121 (15.9)</td>
<td></td>
</tr>
</tbody>
</table>

* Base peak on which percentage abundance was calculated
LC-MS

At a retention time of 23.34 min. a peak of MH\(^+\) 301 was detected. This was consistent with a compound of 300 amu. (fig. 3.20).

Results from GC-MS and LC-MS indicated that the compound was a monohydroxy dimethoxy isoflavanone. It would have been desirable to determine the specific locations of these substituents but unfortunately there was insufficient material for NMR.
Figure 3.18 - Ultraviolet absorption spectrum of isoflavone 1 in methanol scanned from 320 nm to 250 nm. Absorption maxima were 277 nm and 311 nm.
Figure 3.19 - Electron impact mass spectrum of TMS ether derivative of isoflavanone 1 (7,4'-dimethoxy-2'-hydroxyisoflavanone).
Figure 3.20 - Identification of isoflavone 1 (7,4'-dimethoxy-2'-hydroxy isoflavone) in groundnut foliage infected by C. arachidicola by LC-MS. Components were separated by reverse phase HPLC as shown by the total ion chromatogram (top) with ion chromatogram at m/z 301 (centre) and a mass spectrum (bottom) of the peak at 23.34 min.
Isoflavanone 2

This compound was identified as 7-2' dihydroxy-4'methoxy isoflavanone by a combination of GC-MS, LC-MS and HPLC with diode array detection.

**GC-MS**

GC-MS of the trimethyl silyl ether of the authentic material gave m/z = 430 (M+) with prominent peaks at 415 (M+ - 15), 281 (M+ - 149), 222 (M+ - 208) and 207 (M+ - 223) as shown in fig. 3.21.

GC-MS studies with authentic material and extracts from infected material indicated that this compound was present (table 3.8).

**TABLE 3.8**
Comparison of retention time and fragment ions in TMS derivatives of authentic 7,2'-dihydroxy-4'-methoxy isoflavanone and an extract of groundnut foliage infected with *C. arachidicola*.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>STANDARD</th>
<th>INFECTED EXTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>t_R (min.)</td>
<td>28.06</td>
<td>28.14</td>
</tr>
<tr>
<td>Major Ions [m/z (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>430 (23.8)</td>
<td>430 (19)</td>
<td></td>
</tr>
<tr>
<td>415 (7.9)</td>
<td>415 (6.2)</td>
<td></td>
</tr>
<tr>
<td>282 (9.0)</td>
<td>282 (5.8)</td>
<td></td>
</tr>
<tr>
<td>281 (35.2)</td>
<td>281 (20.2)</td>
<td></td>
</tr>
<tr>
<td>224 (6.0)</td>
<td>224 (6.3)</td>
<td></td>
</tr>
<tr>
<td>223 (19.2)</td>
<td>223 (20.7)</td>
<td></td>
</tr>
<tr>
<td>222 (100.0)*</td>
<td>222 (100)</td>
<td></td>
</tr>
<tr>
<td>207 (70.3)</td>
<td>207 (57.4)</td>
<td></td>
</tr>
<tr>
<td>181 (6.7)</td>
<td>181 (7.1)</td>
<td></td>
</tr>
</tbody>
</table>

* Base peak on which percentage abundance was calculated
LC-MS
LC-MS gave a peak at retention time 15.14 min. with MH^- 287 (fig. 3.22)

HPLC (diode array detection)
Authentic material cochromatographed with a peak in an extract from infected leaves which had a similar UV spectrum with absorption maxima at 275 nm and 311 nm. Superimposition of the standard with the unknown, normalised at 275 nm (fig. 3.23) showed a slight difference but this was due to the differences in concentration of the standard and the unknown and interference in the extract.
Figure 3.21 - Electron impact mass spectrum of TMS ether derivative of authentic isoflavanone 2 (7-2'-dihydroxy-4'-methoxy isoflavanone).
Figure 3.22 - Identification of isoflavanone 2 (7,2'dihydroxy-4'methoxy isoflavanone) in an extract of infected groundnut leaves separated by reverse phase HPLC as shown by the total ion chromatogram (top). An ion chromatogram at m/z 287 (centre) shows two peaks of different polarities. On the basis of retention time compared to medicarpin the more hydrophobic component was identified as isoflavanone 2. A mass spectrum of the peak at 15.14 min. (bottom) indicates the purity of the peak.
Figure 3.23 - Comparison of UV spectra from the diode array of authentic 7-2'-dihydroxy-4'-methoxy isoflavanone and an unknown of similar retention time. Spectra were superimposed and normalised at 275 nm. Slight differences were due to background interference in the extract from infected material.
Isoflavonone 3

This was the predominant component in the 50% ethyl acetate fraction eluted from the flash chromatography column and was easily purified by reverse-phase semi-preparative HPLC.

The absorbance maxima in methanol were 278 nm and 311 nm. (fig. 3.24). The compound was more polar than demethylmedicarpin with associated UV spectra having absorbance maxima of 275 nm and 311 nm when analysed by HPLC with diode array detection.

GC-MS

GC-MS of the TMS derivative gave m/z = 430 (M⁺) with prominent peaks at 415 (M⁺ - 15), 282 (M⁺ - 148), 281 (M⁺ - 149), 223 (M⁺ - 207), 222 (M⁺ - 208), 207 (M⁺ - 223) and 191 (M⁺ - 239). Initial results were supported by further studies using the purified material as a standard for comparison with infected extracts (table 3.9).

Electron impact spectra were also obtained by direct probe insertion providing further evidence that the molecular mass for this compound was 286 amu. Major ions obtained were m/z = 286 (11.6%), 271 (16.6%), 270 (100%), 269 (42%), 255 (21%), 150 (19.6%), 149 (6.7%), 148 (15%), 147 (11%) and 137 (25%).
TABLE 3.9

Comparison of retention times and major ions produced by electron impact ionisation of TMS derivatives of the purified standard and crude extracts from infected leaves.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Major Ions [m/z (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STANDARD</td>
</tr>
<tr>
<td>t_R (min.)</td>
<td>30.57</td>
</tr>
<tr>
<td>430 (11.6)</td>
<td>430 (7.2)</td>
</tr>
<tr>
<td>415 (9.5)</td>
<td>415 (5.7)</td>
</tr>
<tr>
<td>282 (5.7)</td>
<td>282 (13.2)</td>
</tr>
<tr>
<td>281 (100)*</td>
<td>281 (32.2)</td>
</tr>
<tr>
<td>223 (19.5)</td>
<td>223 (17.3)</td>
</tr>
<tr>
<td>222 (100)*</td>
<td>222 (100)</td>
</tr>
<tr>
<td>207 (35.0)</td>
<td>207 (85.9)</td>
</tr>
<tr>
<td>191 (6.2)</td>
<td>191 (10.7)</td>
</tr>
</tbody>
</table>

* Base peak on which percentage abundance was calculated

LC-MS

A component at retention time of 11.86 min. had m/z 287 consistent with a dihydroxy-monomethoxy isoflavanone (fig. 3.25)
Figure 3.24 – Methanolic UV spectrum of purified isoflavanone 3 with absorption maxima at 278.4 nm and 311.3 nm (Philips UV/VIS spectrophotometer was used).
Figure 3.25 - Identification of isoflavanone 3 in extract of infected groundnut foliage by thermospray LC-MS. Components were separated by reverse phase HPLC as shown by the total ion chromatogram (top). Mass chromatogram at m/z 287 gave two peaks (centre), the more hydrophobic component was identified as isoflavanone 2 (pages 79-82). The component eluted at 11.86 min. had a mass spectrum with one major ion at m/z 287 (bottom). The MH+ is consistent with the compound being a dihydroxy-monomethoxy isoflavanone.
Daidzein

As with isoflavanone 2 this compound was not isolated but identified using analytical HPLC with diode array detection, GC-MS and LC-MS.

GC-MS

Similar fragment ions were found at the same retention time in both the standard and extract from infected leaves (table 3.10).

**TABLE 3.10**

Comparison of major ions from electron impact spectra of TMS derivatives of authentic daidzein and a compound of similar retention in a sample from infected groundnut leaves.

<table>
<thead>
<tr>
<th>Major Ions [m/z (%)]</th>
<th>STANDARD</th>
<th>INFECTED MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>t_R (min.)</td>
<td>32.30</td>
<td>32.80</td>
</tr>
<tr>
<td>399 (35.5)</td>
<td>399 (33.6)</td>
<td></td>
</tr>
<tr>
<td>398 (100)*</td>
<td>398 (100)</td>
<td></td>
</tr>
<tr>
<td>384 (22.7)</td>
<td>384 (23.8)</td>
<td></td>
</tr>
<tr>
<td>383 (64.8)</td>
<td>383 (70.9)</td>
<td></td>
</tr>
<tr>
<td>281 (9.0)</td>
<td>281 (8.8)</td>
<td></td>
</tr>
<tr>
<td>207 (20.9)</td>
<td>207 (20.1)</td>
<td></td>
</tr>
<tr>
<td>184 (10.7)</td>
<td>184 (14.1)</td>
<td></td>
</tr>
</tbody>
</table>

* Base peak on which percentage abundance was calculated

LC-MS

A peak at retention time 9.39 min. had MH+ 255 consistent with a mass of 254 amu. (fig. 3.26).

HPLC with diode array detection

Absorbance maxima were 245 nm and 299 nm. (fig. 3.27) in
authentic material and a peak of similar retention time in a sample from infected leaves. Differences in the spectra below 230 nm were attributed to background interference in the sample from extracted leaves.
Figure 3.26 - Identification of daidzein in extracts of infected groundnut foliage by LC-MS. Components were separated by reverse phase HPLC as shown by the total ion chromatogram (top). An ion chromatogram at m/z of 255 showed two peaks (centre). The peak at retention time of 9.39 min. was identified as daidzein on the basis of its retention time relative to medicarpin (see appendix 1). This component had a mass spectrum with a major peak at m/z 255.
Figure 3.27 - Comparison of UV spectra from authentic daidzein and a peak of similar retention time in an extract from infected leaves.
Minor antifungal compounds

Two other compounds were isolated or identified by on-line chromatographic spectroscopic techniques as presented for daidzein and isoflavanone 2.

7,2',4'-trihydroxy isoflavanone

The forth isoflavanone was identified by HPLC with diode array detection, GC-MS and LC-MS.

This polar compound was eluted before daidzein and had a UV spectrum with absorbance maxima at 278 nm and 309 nm similar to known isoflavonones.

EI mass spectrum of the trimethylsilyl ether after separation by GC gave m/z = 488 (M+) with prominent peaks at 473 (M+ - 15), 430 (M+ - 58), 267 (M+ - 221), 219 (M+ - 269), 207 (M+ - 281), 164 (M+ - 324), 149 (M+ - 339) and 121 (M- - 367).

LC-MS gave MH+ of 273 at a retention time of 6.2 min.

Trans-trans,2-4 decadienal

This was the main antifungal component of the 100% cyclohexane fraction from the flash chromatography column and was separated from an impurity of similar characteristics by normal phase semi-preparative HPLC.

The Rf. value on TLC was 0.87 and when sprayed with phenylenediamine a brown derivative was produced indicative of the presence of a carbonyl group.

UV absorbance in diethyl ether showed absorbance maxima at 264 nm.

A molecular mass of 152 amu. was obtained by chemical ionisation (CI) at The London School of Pharmacy.

Proton NMR gave a spectrum indicative of an α-β unsaturated aldehyde, especially when chemical shift values of similar compounds identified in wheat leaves were compared (table 3.11).
TABLE 3.11
Proton NMR data obtained for trans-trans, 2-4 decadienal compared to data for triticenes from wheat leaves (Spendley et al., 1982)

<table>
<thead>
<tr>
<th>X</th>
<th>α Triticene</th>
<th>β Triticene</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.513d</td>
<td>9.48d</td>
<td>9.58d</td>
</tr>
<tr>
<td>6.05dd</td>
<td>6.08d</td>
<td>6.13q</td>
</tr>
<tr>
<td>7.06dd</td>
<td>7.29m</td>
<td>7.70q</td>
</tr>
<tr>
<td>6.24-6.29</td>
<td>6.40q</td>
<td>6.32t</td>
</tr>
<tr>
<td>2.19d/q</td>
<td>2.25q</td>
<td>2.40q</td>
</tr>
</tbody>
</table>

* Hydrophobic compound purified from extracts from infected groundnut leaves, later identified as trans-trans, 2-4 decadienal.
DISCUSSION

Conventional extraction was successful for obtaining at least seven antifungal compounds from infected groundnut leaves which could be separated by TLC. Solvent systems developed using TLC were adapted for use in flash chromatography facilitating preparative separations. Although it was possible to collect many 10 ml fractions and obtain some pure compounds without a need for further treatment, it was more practical to collect 50 ml fractions which contained between one and six compounds. These could be readily separated by a simple semi-preparative HPLC method. The advantages of this two-stage method were that yields were maximized, the HPLC column life was extended due to reduced complexity of samples and there was no need for a complicated, time consuming gradient.

As in previous work, the pterocarpan medicarpin was the major phytoalexin produced in groundnut leaves during infection by foliar pathogens (Strange et al., 1985). Most of the other antifungal compounds identified were isoflavonoid phytoalexins often found to accumulate with medicarpin or maackiain. The exception being isoflavanone 1 (dimethoxy-hydroxy isoflavanone) where confirmation of substituents was not obtained. However the results indicate that this compound is similar to isosatvanone isolated from Medicago rugosa (Ingham, 1982).

Dewick (1975) demonstrated that 2',4',4-trihydroxy chalcone (isoliquiritinigenin) was a precursor of formononetin and that both were efficient precursors of medicarpin using CuCl₂ treated seedlings of red clover (Trifolium pratense L.). Daidzein was an inefficient precursor of medicarpin in the same experiments but Wengenmayer et al. (1973) isolated an enzyme that catalysed the conversion of daidzein to formononetin via 4'-methylation using chickpea (Cicer arietinum).

Further work on medicarpin biosynthesis in red clover demonstrated that 7,2'dihydroxy-4'-methoxy isoflavone and 7,2'-dihydroxy-4'-methoxy isoflavanone (vestitone) were also excellent precursors of medicarpin (Dewick, 1977). In these studies the isoflavone had not in fact been identified in treated extracts but complementary studies with white
clover (*Trifolium repens*) showed this compound to accumulate with other precursors of medicarpin (Woodward, 1981). The results from the studies with red clover indicated that medicarpin may be produced by aryl migration with 4' methylation to give formononetin which is 2' hydroxylated, followed by reduction to vestitone. The vestitone is further reduced to an isoflavanol which on loss of water cyclises to form medicarpin. This pathway was supported by the results from similar studies with lucerne (*Medicago sativa*) which also indicated the role for the involvement of a carbonium ion since medicarpin and the isoflavon vestitol were shown to be interconvertible (Dewick and Martin, 1979a; 1979b).

These results are supported by studies on the enzymes involved in biosynthesis. In the last few years many of the enzymes catalysing the later stages in medicarpin biosynthesis have been characterised (Tiemann *et al*., 1987; Hinderer *et al*., 1987; Blä and Barz, 1988).

It is highly likely therefore that formononetin, vestitone and daidzein identified in extracts from infected groundnut leaves are precursors of medicarpin as shown in figure 3.28. Since no isoliquiritigenin was detected in the present study it may be that a 4'methylation of daidzein is more common in this system although other pathways may not be discounted without further investigation.

It is unlikely that demethylmedicarpin is an alternative precursor for medicarpin but it cannot be ruled out without additional biosynthetic studies of this system. Demethylmedicarpin has been demonstrated as a precursor for more complex pterocarpans such as glyceollins (Banks and Dewick, 1983) and phaseollin (Woodward, 1980). In these pathways it was shown that a precursor of demethylmedicarpin was 7,2'4'-trihydroxyisoflavone and this compound was detected in extracts of infected groundnut leaves indicating the existence of possible alternative biosynthetic pathways.

In contrast, demethylmedicarpin by analogy with the demethylation of pisatin (VanEtten *et al*., 1989) and comparison with the modification of medicarpin by the fungus *Aschochyta rabiei* (Kraft *et al*., 1987) is possibly a degradation product. Time course studies on accumulation of major phytoalexins and subsequent experiments with leafspot pathogens grown in media supplemented with medicarpin are discussed.
A third isoflavanone which was not identified would probably not be an isomer of isoflavanone 2 (vestitone) since they were easily separated by TLC, flash chromatography and reverse phase HPLC. However, electron impact mass spectra of derivatised and underivatised compounds gave results identical to those obtained for isoflavanone 2. It seems likely that this compound was a dihydroxymonomethoxy isoflavanone but the positions of substituents is uncertain. However, if retro-Diels-Alder (RDA) fragmentation was the major fragmentation route it would suggest that one hydroxy group was on ring A and the second hydroxy and the methoxy group were on ring B (Dewick, 1982). Further analysis by NMR and chemical modification are necessary.

The only antifungal compounds which were not isoflavonoids were the two hydrophobic compounds extracted in 100% cyclohexane fraction from flash chromatography, one of which was identified as trans-trans, 2,4 decadienal. This compound is often found as a flavour component in plant tissues and roasted groundnut kernels (Johnson et al., 1971), and is produced by the oxidation of linoleic acid (Ohloff et al., 1973). The unsaturated aldehyde was not present in healthy leaves and it was not always present in infected leaves. Hence its involvement in host-pathogen interactions was not investigated any further in this study.

In conclusion the major antifungal compounds isolated from, or identified by on-line spectroscopic techniques, from infected groundnut leaves were isoflavonoids characteristic of many legumes (Ingham, 1982). Their role in resistance of groundnuts to leafspot pathogens was investigated and these experiments are discussed in the succeeding chapters.
Figure 3.28 - Postulated pathway for the biosynthesis of medicarpin in groundnut. Daidzein (1) may be 4'-methylated to give formononetin (2). 2' hydroxylation results in the formation of 7,2'-dihydroxy-4'-methoxyisoflavone (3) which is reduced to 7,2'-dihydroxy-4'-methoxyisoflavanone (4). It has been proposed that vestitone may be further reduced to the isoflavanol (5) which could lose water leading to the formation of a carbonium ion (6) which could cyclize to form medicarpin (7).
Chapter 4

Analytical Method for Determination of Phytoalexins in Groundnut Foliage

INTRODUCTION

Isoflavonoid phytoalexins have been successfully extracted by droplet diffusate methods (Harborne and Ingham, 1978; Ingham, 1981), homogenisation and the facilitated diffusion method (Keen, 1978).

During the early part of this project, liquid-liquid extraction was used to obtain phytoalexins from infected leaves/leaf diffusates or leaves treated with abiotic agents. Although this is a well tried and efficient method it has several major disadvantages for extracting phytoalexins from large numbers of samples. These include the use of large volumes of solvents for repeated partitioning steps with the subsequent need for removal by rotary evaporation.

More recently both normal and reverse phase solid phase extraction (SPE) have been used for the extraction of phytoalexins (Moesta and Grisebach, 1982; Lyon, 1984; Lee et al., 1986). This technique allows selective extraction of solutes onto a solid phase support allowing sample concentration as well as partial purification. Although there are numerous commercial SPE products covering a range of selectivities including ion exchange, normal and reverse phase on the market, a homemade variety with commercially available C18 packing was used in these studies. These have been successfully used for the extraction and concentration of stilbene phytoalexins from groundnut kernels (Cooksey et al., 1988).

Once an efficient extraction procedure had been developed a suitable separation was required where rapid identification was possible. Although several methods had already been devised using TLC, GC/GC-MS and HPLC (Woodward, 1982; Koster et al., 1983, Patroni et al., 1982 and Dziedzic and Dick, 1982) for the separation of isoflavonoids an improved method was needed to separate some of the more complex extracts from infected groundnut leaves.

The column was calibrated with known amounts of phytoalexins
which had been purified and quantified as described in the previous chapter. Calibration curves were obtained for the compounds in order to determine if there was a linear response within a given range of concentrations. As linearity was achieved it was possible to calculate response factors which could be used to enable rapid quantification of components in subsequent analyses.
MATERIALS AND METHODS

Chemicals

Medicarpin was purified from infected groundnut leaves as described in chapter 3. EtOH and EtOAc were general purpose reagents (BDH, Poole). Acetic acid was analytical reagent and acetonitrile and methanol were HPLC grade (Fisons). Pure water was obtained from an ELGA pure water system.

EXTRACTION

Liquid-Liquid Extraction (method 1)

Isoflavonoid phytoalexins were extracted by partitioning into a more hydrophobic organic phase such as ethyl acetate as described in chapter 3.

Solid Phase Extraction (method 2)

The cartridge consisted of 500 mg Techoprep C18 (25-40 μm; HPLC Technology, Macclesfield) held in place by a glass wool plug at either end in a Gilson P1000 tip. The cartridges were fitted into a Supelco vacuum manifold which permitted uniform sample application (up to twelve samples could be processed at any one time). The cartridge was conditioned with methanol and excess methanol was removed by washing with water (fig. 4.1).

Preliminary evaluation of recovery of isoflavonoids

Dried groundnut foliage infected with C. arachidicola was extracted in aqueous ethanol as described in chapters 2 and 3. The sample was taken to dryness by rotary evaporation at 50°C. The residue was taken up in 30% CH₃CN (5 ml) and applied to conditioned cartridges. The cartridges were washed with an additional 5 ml of 30% CH₃CN and phytoalexins were eluted in 100% CH₃CN (1 ml). Samples were analysed by HPLC. Recovery was examined by comparison of detector response for crude extract and for the components eluted from the minicolumn. As separation of all components had not been
optimised at this time an isocratic system was used (Strange et al., 1985). Peak height was used as the quantitative parameter since not all components were well separated.

Recovery of medicarpin by liquid-liquid (method 1) and solid phase extraction (method 2)

Since the phytoalexins were initially extracted in aqueous ethanol samples were diluted to 25% ethanol before application to the conditioned cartridge. Efficiency of this technique was examined by spiking samples with known concentrations of medicarpin.

Groundnut leaves from 6-8 week old plants were removed and 1 g samples were treated with droplets of 10^{-3} M AgNO_3 (10 x 5 µl per leaflet) and incubated at 26°C for 48h. From a total of twelve samples six were spiked with medicarpin (100 µg/sample). Samples (three spiked and three unspiked) were extracted by liquid-liquid extraction and the remaining six were extracted by solid phase extraction. In this experiment the cartridge was eluted with a second ml of CH_3CN and this was analysed separately. Unretained material was concentrated in vacuo and analysed for the presence of medicarpin.
Figure 4.1 - Diagrammatic representation of solid phase extraction as used for extraction of phytoalexins accumulated in infected groundnut foliage.

The cartridge was conditioned with methanol (2 ml) followed by water (2 ml). The 25% ethanol extract containing phytoalexins was applied to the cartridge at a rate of approximately 1 ml/min. The cartridge was washed with 5 ml 25% ethanol to ensure all the more polar components were removed before eluting phytoalexins in acetonitrile (1 ml).
Separation of phytoalexins

Although initial work had involved the use of normal phase HPLC a reverse phase system was selected for developing a routine analytical method as this offered greater flexibility, stability of stationary phase and hence reproducibility.

A Philips apparatus was used, consisting of two pumps (PU 4100), an automatic sampler (PU 4700) and a diode array detector (PU4021) interfaced to a Trivector data system (Philips Analytical, Cambridge, UK). Compounds were separated on a column of Spherisorb ODS1 (250 x 4.6 mm i.d.; 10 µm particle size) protected by an Upchurch low volume guard column (20 mm x 2 mm i.d.; Anachem, Luton UK). The mobile phase was a gradient of acetonitrile in 1% acetic acid in which the acetonitrile concentration was increased from 35% to 40% over the first 12 min and then to 75% over the next 23 min. A reequilibration time of 5 min was allowed to elapse before the next sample was injected. The flow rate was 1.5 ml/min. and the eluent was monitored at 290 nm at an absorbance range of 0.04 AUFS.

Column efficiency was prolonged by a cleaning protocol involving the use of progressively more hydrophobic solvents. Methanol was applied to the column at a flow rate of 1 ml/min. for 30 min. followed by ethyl acetate and then hexane under the same conditions. The protocol was reversed to return to methanol.

Identification

Compounds were routinely identified on the basis of their retention time compared to authentic compounds which were included at the beginning and end of each run of samples. Periodically UV spectra of analytes would be examined as described in chapter 3.

Quantification of Phytoalexins

Since the amounts of phytoalexins purified were low the most accurate way to determine their concentration was by measuring absorbance at their maxima and calculating their concentration using the appropriate molar extinction coefficient as described in chapter.
3. Once the concentration of a particular standard was calculated stock solutions were made up for calibration purposes. The HPLC was calibrated by external standardisation using peak area. A range of concentrations of each phytoalexin were run in order to determine linearity of detector response. If a linear detector response was obtained it was feasible to generate response factors according to the equation below:

\[
\text{Response factor} = \frac{\text{Concentration}}{\text{Area}}
\]

The response factor can then be used for quantification of that component in subsequent analyses. For each phytoalexin many response factors were obtained and a mean response factor was tested for accuracy by using them to quantify a range of concentrations of standards.
RESULTS

Preliminary evaluation of phytoalexin recovery using solid phase extraction (method 2)

Known samples were applied to cartridges and unretained components and those eluted in 100% CH₃CN were examined by analytical HPLC. The main components of interest, those with similar polarity to medicarpin, were retained on the minicolumn when applied in 30% acetonitrile and were eluted by 100% acetonitrile (table 4.1).

TABLE 4.1 - Recovery of compounds by solid phase extraction

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>Rt.* (seconds)</th>
<th>% RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>151</td>
<td></td>
<td>73.32 ± 16.72(^a)</td>
</tr>
<tr>
<td>176</td>
<td></td>
<td>97.42 ± 2.29</td>
</tr>
<tr>
<td>196</td>
<td></td>
<td>85.18 ± 15.96</td>
</tr>
<tr>
<td>220</td>
<td></td>
<td>75.16 ± 10.95</td>
</tr>
<tr>
<td>250</td>
<td></td>
<td>109.13 ± 2.50</td>
</tr>
<tr>
<td>280</td>
<td></td>
<td>96.91 ± 16.02</td>
</tr>
<tr>
<td>339 (medicarpin)</td>
<td></td>
<td>103.49 ± 3.20</td>
</tr>
</tbody>
</table>

* Analysis by reverse phase HPLC was isocratic i.e. acetonitrile: water (1:1 v/v) and at this stage only medicarpin had been identified.

\(^a\) All results were mean of duplicates ± standard deviation
Recovery of medicarpin by liquid-liquid (method 1) and solid phase extraction (method 2)

The modified procedure where the ethanolic extract was applied was as successful as the more conventional liquid-liquid extraction (table 4.2) and any pigments extracted by facilitated diffusion were not eluted by the acetonitrile. Less than 5% of the medicarpin was eluted in the second ml of CH₃CN. No medicarpin was detected in the unretained sample.
### TABLE 4.2 - Recovery of medicarpin by liquid-liquid (method 1) and solid phase extraction (method 2).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>MEDICARPIN (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liquid - Liquid extraction</strong></td>
<td></td>
</tr>
<tr>
<td>1- unspiked*</td>
<td>12.55 ± 4.79</td>
</tr>
<tr>
<td>2- spiked*</td>
<td>114.71 ± 24.68</td>
</tr>
<tr>
<td><strong>Solid phase extraction</strong></td>
<td></td>
</tr>
<tr>
<td>1- unretained eluate (unspiked)</td>
<td>0.00</td>
</tr>
<tr>
<td>2- unretained eluate (spiked)</td>
<td>0.00</td>
</tr>
<tr>
<td>3- Elution 1ml CH₃CN (unspiked)</td>
<td>22.74 ± 5.57</td>
</tr>
<tr>
<td>4- Elution 1ml CH₃CN (spiked)</td>
<td>120.98 ± 5.65</td>
</tr>
<tr>
<td>5- Elution with 2nd ml (unspiked)</td>
<td>0.00</td>
</tr>
<tr>
<td>6- Elution with 2nd ml (spiked)</td>
<td>3.99 ± 3.92</td>
</tr>
</tbody>
</table>

* All values are the means of three replicates ± standard deviation.
* Unspiked samples were extracts of leaves which had been treated with silver nitrate (see text for details).
* Spiked samples were extracts of leaves which had been treated with silver nitrate and spiked with 100 µg of medicarpin.
Separation

A reasonable separation was achieved (fig. 4.2) using a gradient over 35 min. and after five minutes of pumping the solvents of original composition the column was equilibrated and ready for the next sample. To ensure that each peak represented one compound UV spectra throughout the peaks were analysed.

Quantification

A linear detector response was obtained when samples of medicarpin at different concentrations were injected (fig. 4.3). Similar results were obtained for other phytoalexins (data not shown). These results validated the use of response factors which are single point calibrations.

Response factors were calculated for the major phytoalexins where pure standards were available (table 4.3).

TABLE 4.3
Response factors used for phytoalexin quantification

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>RESPONSE FACTOR ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>medicarpin</td>
<td>0.005823 ± 1.98 x 10^-4</td>
</tr>
<tr>
<td>demethylmedlcarpin</td>
<td>0.006146 ± 5.28 x 10^-5</td>
</tr>
<tr>
<td>isoflavanone 1</td>
<td>0.006360 ± 2.47 x 10^-4</td>
</tr>
<tr>
<td>formononetin</td>
<td>0.003510 ± 1.19 x 10^-4</td>
</tr>
<tr>
<td>daidzein</td>
<td>0.002251 ± 4.12 x 10^-5</td>
</tr>
</tbody>
</table>

The response factor obtained for isoflavanone 1 was used to quantify isoflavanones 2 and 3 since all these compounds would have similar extinction coefficients.
Figure 4.2 - Separation of major isoflavonoid phytoalexins in groundnut leaves infected by *Cercospora arachidicola*.

(a) Chromatogram of six phytoalexin standards; a 10 µl sample containing the following concentrations of phytoalexins was injected: 1 - daidzein, 50 µg/ml; 2 - demethylmedicarpin, 25 µg/ml; 3 - 7,2'dihydroxy-4'methoxy isoflavanone, 10 µg/ml; 4 - formononetin, 150 µg/ml; 5 - isoflavanone 1 (7,4'dimethoxy-2'methoxy isoflavanone), 65 µg/ml; 6 - medicarpin, 100 µg/ml.

(b) Typical chromatogram of an extract from infected groundnut leaves with peaks labelled as Fig.2(a).
Figure 4.3 - Calibration curve for medicarpin with concentration ranging from 10 - 100 µg/ml (since injections were 10 µl the on-column concentrations were 100-1000 ng).

Each point was the mean of three replicates, each originating from a different stock solution of the same concentration.
DISCUSSION

Solid phase extraction was an attractive alternative to liquid-liquid extraction. It was possible to process twelve samples at a time, taking about an hour from start to finish with the sample ready for HPLC analysis. Other advantages included the avoidance of using large volumes of solvents and the reduction in sample transfer where losses in recovery may occur. Although a small amount of medicarpin was detected in the second millilitre of acetonitrile, <4%, the good recoveries in the first millilitre of eluate were appropriate for routine work. Although solid phase extraction proved a viable option there were no experiments examining the recovery of other phytoalexins. For a fully validated analytical method this data would need to be obtained.

It would have been desirable to develop an internal standard to assess the efficiency of extraction continually but the variation in minor components in extracts dictated that using the analytical system described the chromatography would be unreliable if a very long gradient wasn't used. This would probably be more practical with the development of the more efficient bonded stationary phases.

Although reasonable separation was achieved on Spherisorb ODS 1 (10 µm), the use of a smaller particle size and a fully endcapped stationary phase would enhance resolution.

The isoflavonoids gave a linear detector response over a practical range of concentrations (100-1000 ng on column) thereby facilitating the use of response factors for quantification. Since all injections were by autosampler and several major phytoalexins were available at >95% purity, external standardisation was the most precise method of quantification.
Chapter 5

Accumulation of Phytoalexins in Groundnuts Infected with C. arachidicola and P. arachidis in the Greenhouse

INTRODUCTION

Once the major phytoalexins were identified it was necessary to investigate their accumulation in cultivars with different susceptibilities to foliar pathogens. Rust and early leafspot were investigated since as a biotroph and a necrotroph, respectively, they differed in their mode parasitism. Resistance of groundnuts to these pathogens has been extensively monitored in the field. Resistance to rust is characterised by increased incubation period, reduced infection frequency, reduction of pustule diameter, percentage pustules ruptured and percentage of leaf area damaged (Subrahmanyan et al., 1985). Germination and penetration occurred irrespective of whether a genotype was immune, resistant or susceptible to rust. In immune species the fungus died shortly after entering the substomatal cavity. Differences in resistance were associated with different rates of fungal development within the leaf (Subrahmanyan et al., 1983).

Cultivars with some resistance to C. arachidicola develop fewer lesions, have reduced sporulation and percentage of lesions sporulating. Resistant cultivars have longer latent periods i.e. the number of days between inoculation and appearance of visual symptoms and increased time to defoliation (Johnson et al., 1986).

In cases of resistance to rust or early leafspot it is possible that one or several plant defence mechanisms are involved once the pathogen has penetrated the leaf tissue.

Early work had shown that there were significant differences in the concentrations of medicarpin in different groundnut cultivars infected with C. arachidicola and P. arachidis (Strange et al., 1985). In systems where phytoalexins were shown to be involved in resistance, such as in certain soybean cultivars to certain races of
Phytophthora megasperma f.sp. glycinea, the phytoalexins reached ED\textsubscript{so} concentrations a few hours after infection and restricted the growth of the fungus (Ebel and Grisebach, 1988). It was therefore necessary to determine the rate and range of phytoalexin accumulation in cultivars of different susceptibilities in relation to disease progress.

This chapter is a comprehensive account of isoflavonoid phytoalexin response in groundnut leaves of two cultivars, Egret and P84/5/244, grown in the greenhouse, to infection by Cercospora arachidicola and Puccinia arachidicola. These cultivars had been shown to have different responses to infection by leafspot pathogens in the field (Cole, personal communication) and significantly different amounts of medicarpin accumulated when infected by either C. arachidicola or P. arachidicola.
MATERIALS AND METHODS

Plant material

Plants of cultivars Egret and P84/5/244 were grown in a greenhouse in a mixture of John Innes No.1 and sand (1:1) at 25-28°C. The plants were grown during the summer months without supplementary lighting and with daily watering. Red spider mites (Tetranychus urticae) were controlled by 'Phytopack' (Phytoseiulus persimilis; Bunting Biological Control, Essex).

Fungal material

F. arachidis was isolated from a 'hot spot' in southern Texas. Uredospores were removed from leaves by brushing with a camel hair paintbrush and were stored at -20°C until required. C. arachidicola was isolated from infected leaves in Zimbabwe. The leaves were washed in running tap water for five minutes after which infected lesions were excised and immersed in 0.5% sodium hypochlorite for 1 minute. They were subsequently rinsed several times with sterile distilled water and incubated in moist, sterile petri dishes under continuous fluorescent light to induce sporulation. Individual conidia were removed and placed on potato dextrose agar (PDA). The fungus was thereafter maintained on peanut oatmeal agar (Smith, 1971) and incubated under cool white fluorescent light at 26°C. Conidial suspensions were obtained by flooding petri-dishes containing 3-4 week old cultures with distilled water and agitating with a glass rod. Mycelial fragments were removed by passage of the suspension through two layers of muslin.

Inoculation procedure

Spores of each fungus were suspended in 100 ml distilled water \(\left(10^6 \text{ ml}^{-1}\right)\) containing 3 drops of Tween 20 (polyoxyethylene sorbitan monolaurate) and the suspensions were used to inoculate 6 week old plants. Plants were sprayed until the leaf surfaces were just wet and then covered in polythene bags to provide the humid conditions.
necessary for spore germination and infection. The bags were removed after 2 days for rust inoculations and 5 days for *C. arachidicola* inoculations. Rust inoculum was applied in the late afternoon since strong sunlight inhibits their germination (Subrahmanyam *et al.*, 1983; Subrahmanyam and McDonald, 1983).

**Disease assessment**

Rust was assessed visually using the 1-9 scale (Subrahmanyam *et al.*, 1983) and chemically using the chitin assay (Ride and Drysdale, 1972). This involved grinding 0.2 g leaf, cut into strips, with a pinch of sand followed by extraction with acetone. The acetone extract was centrifuged at 1,500 g for 10 min. and the residue mixed with potassium hydroxide solution (120 g/100 ml of water) to give a final volume of 2 ml. The alkaline mixture was incubated at 145°C for 1 hour, after which it was cooled, mixed with 8 ml 75% ethanol and allowed to stand for 15 min in iced water. Celite suspension was placed on the contents of the tube which was centrifuged at 1,500 g for 10 min. The resulting residue was washed with 40% EtOH followed by 2 washes with ice cold water. The final residue was made up to 1.5 ml with water and mixed with NaNO₂ (1.5 ml, 5% solution) and KHSO₄ (1.5 ml, 5% solution). This mixture was shaken at regular intervals for 15 min before centrifugation. Two aliquots of supernatant (1.5 ml each) were removed and to each was added NH₄SO₃NH₂ (0.5 ml, 12.5% solution). Samples were shaken for 5 min before fresh 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) (0.5 ml, 0.5%) solution was added. The mixture was heated in boiling water for 3 min and allowed to cool before FeCl₃ (0.5 ml, 0.5% solution) was added. After standing at room temperature for 30 min. the absorbance at 650 nm was read.

Known amounts of glucosamine were used to obtain a standard curve from which to calculate the amount of fungus present. It was not possible to estimate the amount of glucosamine per gram of fungus since it is an obligate biotroph.

Early leafspot was assessed visually by calculating the average number of lesions per leaflet and by weighing abscised leaves.
Sample preparation

Leaf samples (1 g fresh weight) were harvested at various times after inoculation (7 or 3 day intervals) and in all experiments there were either 3 or 5 replicates. Each sample was extracted in 15 ml 60% EtOH by facilitated diffusion (Keen, 1978) as described in chapter 2. The ethanol was decanted and diluted to 25% before being applied to a solid phase cartridge as described in chapter 4.

Analysis of phytoalexins

Samples were analysed by reverse-phase analytical HPLC with diode array detection as described in chapter 4. Seven phytoalexins were quantified, medicarpin, formononetin, demethylmedicarpin, 7-4'dimethoxy-2'hydroxy isoflavanone (INE 1), 7,2'-dihydroxy-4'-methoxy isoflavanone (INE 2), an unidentified isoflavanone (INE 3) and daidzein their total being that referred to as 'total phytoalexin' in the results.
RESULTS

Infection with *Cercospora arachidicola*

Cultivar Egret was less susceptible than P84/5/244 when either the number of lesions (fig. 5.1a) or the amount of defoliation (fig. 5.1b) was compared. Similar results were obtained from an earlier time course experiment (results not shown). *C. arachidicola* did not contain detectable amounts of chitin, thus chemical estimation of the fungus in leaf tissue was not possible.

Phytoalexins accumulated in both Egret and P84/5/244 as infection progressed whereas only trace amounts of these compounds were detected in uninfected foliage (fig. 5.2a & b). In P84/5/244 total phytoalexins increased linearly 12 days after infection (fig. 5.2a) whereas in Egret phytoalexins accumulated at an exponential rate 9 days after infection (fig. 5.2b) when the first small lesions were visible.

Composition of phytoalexin response

The major phytoalexins in samples from both cultivars were medicarpin, isoflavanone 1 (7,4′-dimethoxy-2′-hydroxy isoflavanone), formononetin and demethylmedicarpin. In P84/5/244 the concentration of medicarpin began to increase 3 days after inoculation but it never exceeded 100 μg/g. f. wt. Formononetin and isoflavanone 1 accumulated in a similar manner to medicarpin. Demethylmedicarpin began to accumulate 12 days after inoculation and was the predominant phytoalexin in all subsequent samples (fig. 5.3a). In Egret, medicarpin began to increase steadily 3 days after inoculation reaching concentrations of >100 μg/g. f.wt. 15 days after inoculation. Formononetin increased at a similar rate and to similar concentrations as medicarpin. Concentrations of isoflavanone 1 increased 9 days after inoculation and were constantly between 50-100 μg/g.f.wt. in subsequent samples. Demethylmedicarpin also began to increase 9 days after inoculation and reached maximum concentrations 27 days after inoculation (fig. 5.3b).
Figure 5.1 - Assessment of infection with *C. arachidicola* in cultivars P84/5/244 (■) and Egret (+) by (a) calculating the average number of lesions and (b) estimating the amount of defoliated leaves per plant.
Figure 5.2 - Accumulation of isoflavonoid phytoalexins in (a) cultivar P84/5/244 and (b) Egret infected by *C. arachidicola* (□) and in uninfected controls (■). Samples (5 x 1 g.) were taken every three days after inoculation at time zero.
Figure 5.3 - Concentration of major phytoalexins, medicarpin (■), formononetin (▲), isoflavanone 1 (+) and demethylmedicarpin (□) in (a) P84/5/244 and (b) Egret.
Infection by *Puccinia arachidis*

The amount of disease in the early stages of infection was similar in both cultivars. However, between 28 and 42 days the amount of disease remained steady in Egret while continuing to increase linearly in P84/5/244 (fig. 5.4).

Chemical estimation (fig. 5.4b) of the amount of fungus during infection was calculated by subtracting values obtained for controls from those for infected leaves. Although chitin was detected in both cultivars, higher concentrations were found in P84/5/244 on most occasions after the first 28 days. Positive results in the uninfected controls were probably due to the presence of interfering compounds reacting with the 3-methyl-2-benzothiazolone (results not shown). This may explain why the correlation between visual assessment and chemical estimation is not complete.

Little or no phytoalexin accumulated in P84/5/244 (fig. 5.5a) compared with concentrations in excess of 250 µg/g.f.wt. in Egret (fig. 5.5b). Uninfected plants of either cultivar contained little or no phytoalexin.

**Composition of phytoalexin response**

In P84/5/244 (fig. 5.6a) only marginally more phytoalexin than in the controls was detected with the main component being medicarpin. In Egret medicarpin, isoflavonone 1 and formononetin increased steadily during infection with little or no demethylmedicarpin (fig. 5.6b).
Figure 5.4 - Assessment of rust in P84/5/244 (■■■) and Egret (■■) by a visual method (a) using the 1-9 scale* and by estimation of chitin (b).

- Categories of 1-9 scale:
  1 - no disease, 2 - few, very small pustules on older leaves, some ruptured, poor sporulation,
  3 - few pustules, mainly on older leaves, mostly on lower or middle leaves, disease evident,
  4 - pustules small and large, mostly on lower and middle leaves, yellowing and necrosis of some lower leaves, moderately sporulating,
  5 - as 4 but pustules heavily sporulating,
  6 - as 5 but pustules heavily sporulating,
  7 - pustules all over plant; lower and middle leaves withering,
  8 - as 7 but withering more severe,
  9 - plants severely affected, 50-100% leaves withering.
Figure 5.5 - Accumulation of phytoalexins in (a) P84/5/244 and (b) Egret infected by *P. arachidis* (■) and in uninfected controls (□). Samples (3 x 1 g.) were taken at weekly intervals one week after inoculation.
Figure 5.6 - Concentration of major phytoalexins, medicarpin (■), formononetin (▲), isoflavanone 1 (+) and demethylmedicarpin (□) in (a) P84/5/244 and (b) Egret during infection by *P. arachidis*. 
DISCUSSION

The greenhouse time course studies were the first detailed analysis of phytoalexin accumulation and disease progression in groundnut foliage. Although the reaction of both pathogens with P84/5/244 and Egret were considered compatible, the degree of compatibility was different. During infection by either *P. arachidis* or *C. arachidicola*, P84/5/244 showed more severe disease symptoms with a slower and lower level of phytoalexin accumulation than Egret.

The amount of phytoalexins produced by both cultivars was higher in interactions with *C. arachidicola* than *P. arachidis*. This could possibly be explained by the mode of nutrition of the two fungi, the biotroph *P. arachidis* causes little cell death in comparison with the necrotroph, *C. arachidicola*. Thus supporting the endogenous elicitor theory (Bailey, 1982) whereby pectic plant cell wall fragments are released as a result of injury, or specific enzymic activity and subsequently activate plant defense mechanisms. Further studies on precise mechanisms of elicitation are necessary to determine the involvement of endogenous elicitors.

As well as differences in amounts of total phytoalexins in plants inoculated with *P. arachidis* and *C. arachidicola* there were also differences in the composition of major compounds. The most notable difference was the presence of demethylmedicarpin in foliage infected *C. arachidicola* and its absence in foliage infected with *P. arachidis*. This may be explained by the fact that demethylmedicarpin is a product of fungal modification of medicarpin and the mode of infection of *C. arachidicola* i.e. one that elicits high concentrations of phytoalexins has necessitated the ability to overcome these compounds. *P. arachidis* does not elicit such concentrations of phytoalexins and thus seems to have no ability to detoxify them. Medicarpin detoxification has been demonstrated in numerous host-pathogen systems in particular that of chickpea (*Cicer arietinum*) infected by *Nectria haematococca* and *Ascochyta rabiei* where 9-O-demethylation is one mode of detoxification (VanEtten et al., 1989). The presence of demethylmedicarpin as in leaves infected by *C.
arachidicola and not in those infected with *P. arachidis*, suggests that this compound is a fungal metabolite rather than a compound synthesized by the plant.

In P84/5/244 infected by *C. arachidicola*, demethylmedlcarpin accumulated to much higher concentrations than did medicarpin. On the other hand in Egret medicarpin was the major phytoalexin although there were high concentrations of demethylmedlcarpin as plants became more severely infected. These observations suggest that in P84/5/244 the pathogen has the advantage because the plant appears to be unable to maintain levels of medicarpin which may retard the spread of the pathogen. In Egret it is possible that the higher levels of medicarpin may have had some effect in restricting spread of the fungus resulting in slower disease progression.

To determine more precisely the role of phytoalexins in the described host-pathogen interactions it would be essential to investigate the accumulation of the phytoalexins at precise locations of fungal development as in other systems including broad bean-*Botrytis* spp. (Mansfield & Hutson, 1980) and Soyabeen-*P.megasperma* (Yoshikawa et al., 1978; Hahn et al., 1985). It would also be essential to investigate reactions between resistant germplasm and a selection of isolates, investigating also the possible involvement of other resistance mechanisms such as lignification, the accumulation of hydroxyproline-rich glycoproteins, chitinases and glucanases.
Localization of Phytoalexins in Groundnut Leaves Infected with *Cercospora arachidicina*

**INTRODUCTION**

It is necessary to measure the concentration of phytoalexins in the vicinity of the pathogen during infection in order to determine their role in it. Such studies have often involved crude dissection followed by TLC and UV spectrometry.

More recently monoclonal antibodies have been produced which are specific to Glyceollin I, the major phytoalexin in infected tissues of soybean (Moesta et al., 1983). This technique was a thousand fold more sensitive than previous methods and it was linear in a range from 1 to 100 picomoles. The complementary use of an immunofluorescent stain for the hyphae of *P. megalosperma f.sp. glycinea* (Pmg) permitted precise localization of the fungus in infected tissues. The combined use of these methods demonstrated that in an incompatible interaction, significant amounts of Glyceollin I were detectable at the sites of infection as early as 5 hours after inoculation. Further the EC\textsubscript{50} value of 0.6 nM was exceeded 8 hours after inoculation (Hahn et al., 1985).

Laser microprobe analysis (LAMMA) was also used for the determination of glyceollin concentrations in situ. This technique was a combination of a laser microscope with a mass spectrometer. The microscope was used for observation of the tissue under investigation and for focusing the UV-pulse laser beam onto a target area (spot size 1 \( \mu \text{m} \) diameter). The laser beam ionized the exposed tissue and resulting ions were detected by the mass spectrometer. Using this technique Moesta et al. (1982) demonstrated the presence of glyceollins I, II and III in 10 \( \mu \text{m} \) thick sections prepared from limited lesions formed in cotyledon tissue inoculated with Pmg but accurate quantitation was not possible.

In order to evaluate the role of phytoalexins it is essential to
determine concentrations at cellular level relative to invading hyphae. However, the techniques described require a great deal of method development for each system and this was not possible within the time span of this work. However, it was possible to determine the location and concentration of the isoflavonoid phytoalexins by crude leaf dissection.
MATERIALS AND METHODS

Infected plant material
Groundnut cultivars Egret, Early Bunch and NC9 were grown in the greenhouse and infected with C. arachidicola as described in chapter 5.

Location of antifungal activity
Several leaves with prominent lesions 4-6 mm diameter were air dried. Dried leaves were immersed in ethyl acetate and blotted on a TLC plate (silica gel 60). The TLC plate was sprayed with a spore suspension of C. cucumerinum and incubated at 25-27°C at high humidity for 48 hours. Plates were then examined for areas of growth inhibition.

Analysis of phytoalexins in infected leaves
Three portions of infected leaves were examined, the lesion, the tissue adjacent to the lesion and uninfected tissue.

A 4 mm cork borer was used to remove disks (2 x 20) of necrotic and healthy tissue from each of the three cultivars selected. An 8 mm cork borer was used to remove tissue adjacent to the lesion once it had been removed. Samples were weighed and extracted in 60% ethanol followed by solid phase extraction as described in chapter 4.

Samples were analysed by analytical reversed-phase HPLC with diode array detection as described in chapter 4 and phytoalexins were identified on the basis of their retention time and UV spectra.
RESULTS

Location of antifungal activity

The crude blotting bioautograph demonstrated that the areas of growth inhibition of the \textit{C. cucumerinum} correlated with the location of the lesions on the leaf indicating that the highest concentrations of phytoalexins were located in the necrotic tissue (results not shown).

Analysis of phytoalexins in leaves infected with \textit{C. arachidicola}

In all three cultivars examined the major phytoalexins were medicarpin, formononetin, isoflavanone 1, demethylmedicarpin, isoflavanone 2 & 3 and daidzein with highest concentrations in the necrotic tissue. Approximately one tenth of the phytoalexin concentration in the necrotic tissue was found in the adjacent healthy tissue and virtually no phytoalexin was found in the healthy tissue (fig. 6.1).

Analysis of composition of phytoalexins in leaves infected with \textit{C. arachidicola}

In all three cultivars the predominant phytoalexin in the necrotic tissue was the pterocarpan, demethylmedicarpin (fig. 6.2). Concentrations of demethylmedicarpin were at least twice that of medicarpin, the second major phytoalexin in the necrotic tissue.

In adjacent tissue the proportions of demethylmedicarpin to medicarpin were similar, in cultivars Egret and Early Bunch medicarpin was the most abundant phytoalexin whereas in NC9, demethylmedicarpin was still the main component (fig. 6.3).

In all three cultivars small amounts of medicarpin were detected in healthy tissue but none of the other phytoalexins were detectable (fig. 6.4).

Although the concentrations of phytoalexins were highest in the lesions the amount per individual lesion was 120-124 \(\mu\)g total phytoalexin in cv Egret much of this comprising demethylmedicarpin.
Figure 6.1 - Localization of phytoalexins in necrotic tissue (section 1), adjacent tissue (section 2) and healthy tissue (section 3) in cultivars Egret, Early Bunch and NC9. Each sample comprised 2 x 20 extracted pieces of tissue (Sample A and Sample B).
Figure 6.1 - Localization of phytoalexins in necrotic tissue (section 1), adjacent tissue (section 2) and healthy tissue (section 3) in cultivars Egret, Early Bunch and NC9. Each sample comprised 2 x 20 extracted pieces of tissue (Sample A and Sample B).
Figure 6.2 – Concentrations of phytoalexins in necrotic leaf tissue from groundnut leaves infected with C. arachidicola in cultivars; Early Bunch ( ), Egret ( ) and NC9 ( ). Values represent the mean of duplicates. Standard deviation was between 2 and 20%.

MED - medicarpin, INE 1 - Isoflavone 1 (7,4'dimethoxy-2'hydroxy isoflavanone), FORM - formononetin, INE 2 - Isoflavone 2 (7,2' dihydroxy-4'methoxy isoflavanone), DEM - demethylmedicarpin, INE 3 - isoflavanone 3 (unidentified dihydroxy monomethoxy isoflavanone) and DAID - daidzein.
Figure 6.3 - Concentrations of phytoalexins in tissue adjacent to the necrotic area of groundnut leaves infected with *C. arachidicola* in cultivars; Early Bunch (■), Egret (■■) and NC9 (■■■). Values represent the mean of duplicates. Standard deviation was between 4 and 25%.

MED - medicarpin, INE 1 - Isoflavanone 1 (7,4'dimethoxy-2'hydroxy isoflavanone), FORM - formononetin, INE 2 - Isoflavanone 2 (7,2' dihydroxy-4'methoxy isoflavanone), DEM - demethylmedicarpin, INE 3 - isoflavanone 3 (unidentified dihydroxy monomethoxy isoflavanone) and DAID - daidzein.
Figure 6.4 - Concentrations of phytoalexins in sections of healthy tissue from groundnut leaves infected with C. arachidicola in cultivars; Early Bunch ( ), Egret ( ) and NC9 ( ). Values are the mean of duplicate samples. Standard deviation was between 3 and 15%.

MED - medicarpin, INE 1 - Isoflavanone 1 (7,4'-dimethoxy-2'hydroxy isoflavanone), FORM - formononetin, INE 2 - Isoflavanone 2 (7,2' dihydroxy-4'methoxy isoflavanone), DEM - demethylmedicarpin, INE 3 - isoflavanone 3 (unidentified dihydroxy monomethoxy isoflavanone) and DAID - daidzein.
DISCUSSION

Although these analyses were crude, the TLC bioassay demonstrated that the antifungal activity, hence the higher concentrations of phytoalexins, were associated with necrotic tissue. These observations were supported by results obtained from leaf dissection where the highest concentrations of phytoalexins were found in the necrotic tissue. Much lower concentrations were found in adjacent tissue and only low concentrations of medicarpin were detected in healthy tissue. Although the cultivars varied in the magnitude of phytoalexins produced, their distribution within the infected leaf was similar. These observations are consistent with others where it was proposed that phytoalexins are synthesized in healthy cells but accumulate in necrotic tissue (Bailey, 1982).

Demethylmedicarpin was the major phytoalexin in the necrotic tissue and medicarpin was the major component in adjacent tissue suggesting that demethylmedicarpin is the result of detoxification of medicarpin by C. arachidicola. The presence of demethylmedicarpin in the tissue adjacent to the lesion was due to the fact that the edges of this tissue were necrotic.

These experiments only provided information regarding the distribution of phytoalexins in peanut leaves infected with C. arachidicola. Thus it is not possible to determine the extent of the antifungal environment encountered by the invading fungus. Further experiments involving leaf dissection with concomitant analysis of phytoalexins and fungus would be required to determine the direct involvement of phytoalexins on fungal growth.
Chapter 7

Accumulation of Phytoalexins in Groundnuts Infected with Foliar Pathogens in the Field

INTRODUCTION

Evidence that phytoalexins were involved in host-pathogen interactions originated from studies on interaction between *C. arachidicola* and *F. arachidicola* (Cole, 1982). Plants are infected with *C. arachidicola* earlier than *F. arachidicola* and these leaves are not susceptible to web blotch. When canopy was not limited the two pathogens spread independently. However, when the canopy was limited *C. arachidicola* increased at the expense of *F. arachidicola*.

Extracts from leaves infected with *C. arachidicola* were inhibitory towards *F. arachidicola*, and this was shown to be due to phytoalexins, principally medicarpin (Strange *et al.*, 1985).

Studies on resistance of genotypes to both pathogens have indicated that successful penetration may occur in both resistant and susceptible cultivars (Ricker *et al.*, 1985; Subrahmanyam and Smith, 1987). In resistant plants the development of the pathogen is retarded resulting in increased latent period i.e. the number of days between inoculation and observation of the first sporulating lesion, smaller lesions with a reduction in the percentage of lesions sporulating and increased time to defoliation.

The results of analysis of phytoalexin production by groundnut leaves infected with *C. arachidicola* and *P. arachidis* in greenhouse grown plants indicated that phytoalexins may be involved in retarding disease (chapter 5). Since it was shown that some cultivars may differ in susceptibility to leafspot pathogens in the field compared to the greenhouse (Hassan and Beute, 1977) it was necessary to examine the response of the same cultivars in the field.

This chapter includes analysis of phytoalexin accumulation in several cultivars of differing levels of susceptibility to the leaf spot pathogens *C. arachidicola* and *P. arachidicola* during four growing seasons in Zimbabwe. Progress of disease was monitored regularly.
throughout all the growing seasons.
MATERIALS AND METHODS

Plant material

Cultivars belonging to two subspecies of *A. hypogaea*, s.sp. *hypogaea* and s.sp. *fastigata* were used in all field trials over four growing seasons (1984/85, 1985/86, 1986/87 and 1987/88). Subspecies *hypogaea* does not have flowers on the main plant stem and has alternating pairs of vegetative and reproductive axes along the lateral branches. Subspecies *fastigata* has flowers on the main plant stem and sequential reproductive axes along lateral branches (Krapovickas, 1968, 1973).

Long season cultivars (alternate branching [s.sp. *hypogaea*]; c. 175 days from planting to maturity) Egret, Flamingo, P84/5/244, 6/11/11 were planted between 27 and 30th September in each growing season. Short season cultivars (sequential branching [s.sp. *fastigata*]; c.140 days from planting to maturity) Jacana, Valencia R2 and Plover, were planted in mid November of each year. Three short season cultivars monitored in 1987/88, Malimba, JL 24 and Natal Common were also planted in mid-November. The mid season cultivar Swallow was planted in the first week of October.

The plants were grown in heavy red clay soil at the University of Zimbabwe, Department of Crop Science field plots (latitude 17.48°S and altitude 1500 m). Groundnuts were irrigated where necessary.

Special treatments

In growing season 4 (1987/88) four plots of the long season cultivar, Flamingo were sprayed with a vandozeb and thiophanate methyl mixture to control early leafspot. The mixture was applied from 7/12/87 to 29/2/88 at fourteen day intervals in 250 L with a knapsack sprayer at a pressure of 275 kPa.

Another four plots of Flamingo were sprayed with the systemic fungicide, procymidine to control web blotch.

Disease assessment

Early leafspot (*Cercospora arachidicola*) and web blotch (*Phoma arachidicola*) were visually assessed on a class scale (Cole, 1981).
(a) Early leafspot was assessed on the following scale:

0 = No disease
1 = Isolated spots on leaflets
2 = Up to 10 leaflets infected, several spots/leaflet, a few leaflets on the ground.
3 = Up to 25% of leaflets in plot spotted, defoliated leaflets more numerous.
4 = Up to 50% of leaflets spotted, marked defoliation on long-season cultivars.
5 = Up to 75% leaflets spotted, most of these on the ground (long-season cultivars) and some defoliation in short-season cultivars.
6 = Plant almost completely defoliated (long-season cultivars), extensively defoliated (short-season cultivars), remaining leaflets all infected.

(b) Web blotch was assessed on the following scale:

0 = No disease
1 = Tiny tan or purplish lesions on lower leaves.
2 = Lesions established, spreading to upper leaves but still discrete.
3 = Lesions large and beginning to merge, covering half the leaflet; infected leaflets scattered throughout the canopy.
4 = Canopy has a dirty appearance.
5 = Most leaflets infected, many dying and falling off.
6 = Plant almost completely defoliated and remaining leaflets brown with blotch.

**Sampling strategy**

The first leaves for phytoalexin analysis were sampled 10 weeks (long season), 4 weeks (mid season) and 3 weeks (short season) after planting to ensure that samples were disease free. This was based on 10 years observations by D. Cole. Subsequently, infected leaves were removed at monthly intervals or in growing season 4 at fortnightly
interval.

In growing seasons 2 and 3 samples were also collected from the healthy upper leaves of plants infected by *C. arachidicola* or *P. arachidicola*.

**Extraction of phytoalexins**

In the first three growing seasons, leaf samples (20 g/f. wt.) were extracted in 60% aqueous methanol (15 ml/g fresh tissue) using facilitated diffusion as described in chapter 2. After approximately 4 hours the methanol extract was decanted and reduced *in vacuo* to a quarter of the volume. This was extracted by liquid-liquid partitioning with ethyl acetate as described in chapter 2.

In the fourth growing season, samples (14 x 2 g replicates) were air dried. They were extracted in aqueous ethanol followed by solid phase extraction as described in chapter 4.

**Analytical HPLC**

Samples were separated by reverse phase HPLC as described in chapter 4. Isoflavonoid phytoalexins were identified by comparison of retention times and UV spectra with those of authentic standards and were quantified by external standardisation as previously described (chapter 3).
RESULTS

Growing season 1 - 1984/85

Infection by C. arachidicola

All four long season cultivars, Egret, Flamingo, 6/11/11 and P84/5/244 were susceptible to early leafspot (fig. 7.1). High disease was not recorded since samples were only taken up to 15th January.

Isoflavonoid phytoalexins were identified in extracts from the infected material and medicarpin was the predominant phytoalexin in most cases. Since some of the compounds identified had been shown to be products of pathogen metabolism (chapter 8) it seemed more realistic to examine medicarpin accumulation as an indication of the plant's response to infection.

Low concentrations of medicarpin were detected in 6/11/11 but there was no change in this as disease progressed (fig. 7.1a). In P84/5/244 medicarpin increased gradually as disease progressed (fig. 7.1b). Medicarpin accumulated to higher concentrations in Flamingo as disease progressed before declining (fig. 7.1c). In Egret concentrations of medicarpin were high but the amount of disease was similar to 6/11/11 where only small amounts of medicarpin were detected (fig. 7.1d).

Similar results were obtained with the short season cultivars Jacana and Valencia R2. Both were susceptible to C. arachidicola but low concentrations of medicarpin were detected in Jacana compared to higher concentrations in Valencia (results not shown since samples were only taken on two occasions).
Figure 7.1 – Severity of leafspot caused by Cercospora (■) and medicarpin accumulation (□) in (a) 6/11/11, (b) P84/5/244, (c) Flamingo and (d) Egret in growing season 1. Sampling times were 1 = 5/12/84, 2 = 18/12/84, 3 = 3/1/85 and 4 = 15/1/85. Each point represents the mean of four replicate plots.
Infection by *Phoma arachidicola*

Cultivar 6/11/11 was most susceptible to *P. arachidicola* and P84/5/244 was least susceptible whilst Egret and Flamingo were intermediate (fig. 7.2)

Traces of medicarpin were detected in 6/11/11 but these did not change as disease progressed (fig. 7.2a). Medicarpin accumulated as disease progressed in P84/5/244 but concentrations did not exceed 50 μg/g.f.wt. (fig. 7.2b). In Flamingo and Egret medicarpin concentrations increased linearly with time (fig. 7.2c & d).

Short season cultivars Jacana and Valencia R2 were susceptible to *P. arachidicola* but as with *C. arachidicola* much lower concentrations of medicarpin were detected in extracts from Jacana compared to Valencia (results not shown).

*Comparison of phytoalexin elicitation by P. arachidicola and C. arachidicola*

For all cultivars the concentrations of total phytoalexin and medicarpin in plants infected with *P. arachidicola* and *C. arachidicola* were compared in order to determine any difference in response. Cultivar Flamingo accumulated higher concentrations of phytoalexins when infected with *P. arachidicola* compared with *C. arachidicola* (table 7.1). Valencia R2 accumulated higher concentrations of phytoalexins when infected with *C. arachidicola*. In Egret, 6/11/11, P84/5/244 and Jacana similar concentrations of phytoalexins were detected when infected by either pathogen.
Figure 7.2 - Severity of leafspot caused by *Phoma* (□) and medicarpin accumulation (■) in cultivars (a) 6/11/11, (b) P84/5/244, (c) Flamingo and (d) Egret. Sampling times were 1 = 17/1/85, 2 = 30/1/85; 3 = 14/2/85 and 28/2/85. Each point represents the mean value of four replicate plots.
TABLE 7.1
Comparison of medicarpin and total phytoalexins in cultivars infected by *C. arachidicola* (C.a.) and *P. arachidicola* (P.a.).

<table>
<thead>
<tr>
<th></th>
<th>TOTAL PHYTOALEXIN</th>
<th>MEDICARPIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/gram fresh weight)</td>
<td></td>
</tr>
<tr>
<td>Egret</td>
<td>551</td>
<td>838</td>
</tr>
<tr>
<td>Flamingo</td>
<td>175</td>
<td>481</td>
</tr>
<tr>
<td>6/11/11</td>
<td>96</td>
<td>51</td>
</tr>
<tr>
<td>P84/5/244</td>
<td>151</td>
<td>249</td>
</tr>
<tr>
<td>Jacana</td>
<td>232</td>
<td>127</td>
</tr>
<tr>
<td>Valencia R2</td>
<td>650</td>
<td>334</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td><strong>309</strong></td>
<td><strong>336</strong></td>
</tr>
</tbody>
</table>

Correlation coefficient (r): 0.5654 0.5699

Values in table represent the mean values of four replicate plots at final samplings in 1984/85 (growing season 1).
GROWING SEASON 2 - 1985/86

Infection by Cercospora arachidicola

All cultivars were susceptible to C. arachidicola. Egret and 6/11/11 were more susceptible than P84/5/244 and Plover (fig. 7.3).

In cultivar 6/11/11 only trace amounts of medicarpin were detected when plants were heavily infected on 20/2/86 (fig. 7.3a). Similar levels of medicarpin were detected in P84/5/244, <10 μg/g.f.wt. (fig. 7.3b).

In contrast in cultivars Plover and Egret medicarpin accumulated more or less linearly with time as did severity of disease (fig. 7.3c & d). No disease assessment data was available for cultivar Flamingo, but medicarpin accumulated to concentrations similar to those detected in Egret (results not shown).

Infection by Phoma arachidicola

The long season cultivars 6/11/11 and Egret were susceptible to P. arachidicola whereas P84/5/244 and Plover demonstrated some resistance (fig. 7.4).

Small changes in concentrations of medicarpin were detected in 6/11/11, P84/5/244 and Plover (fig. 7.4a, b & d) over the growing season. In contrast medicarpin accumulated rapidly in Egret from the end of January (fig. 7.4c).

Although there was no disease assessment data for Flamingo, medicarpin accumulated rapidly between late January and late February as seen for Egret.
Figure 7.3 - Severity of leafspot caused by Cercospora (□) and medicarpin accumulation (■) in (a) 6/11/11, (b) P84/5/244, (c) Plover and (d) Egret. Samples were collected at 1 = 17/12/85, 2 = 22/1/86 and 3 = 20/2/86. Points represent the mean of five replicate plots.
Figure 7.4 - Severity of leafspot caused by *Phoma* (□) and medicarpin accumulation (■) in (a) 6/11/11, (b) P84/5/244, (c) Plover and (d) Egret. Samples were collected at 1 = 17/12/85, 2 = 22/1/86 and 3 = 20/2/86. Points represent the mean of five replicate plots.
Comparison of phytoalexins induced by *C. arachidicola* and *P. arachidicola*

Cultivars 6/11/11 and Egret accumulated higher concentrations of phytoalexins when infected by *P. arachidicola*. In contrast Flamingo and Plover produced more phytoalexins when infected by *Cercospora*. The concentrations of phytoalexins accumulating in all cultivars infected with *C. arachidicola* was poorly correlated with those obtained for the same cultivars infected with *P. arachidicola*. Since correlation of the severity of the two diseases at the time of sampling was not high this may, in part have some effect on the observed concentrations of phytoalexins in some cultivars (data not shown). However, for an overall comparison of phytoalexins and medicarpin induced by the two fungi, mean values showed no significant difference (table 7.2).

Analysis of phytoalexins in samples from healthy leaves from plants infected on the lower leaves with *C. arachidicola*

In all five cultivars sampled isoflavonoid phytoalexins were detected in healthy leaves from the upper part of the plant where only the lower leaves were infected. Concentrations of phytoalexins found in the upper leaves were 62% of the concentration found in the lower leaves (table 7.3).

The major phytoalexins were medicarpin and demethylmedicarpin. In the lower leaves 35% of total phytoalexins was medicarpin and 32% demethylmedicarpin. In the upper leaves 50% of total phytoalexins was medicarpin and 18% demethylmedicarpin.

Analysis of phytoalexins in samples from healthy leaves from plants infected on the lower leaves with *P. arachidicola*.

In all five cultivars, phytoalexins were detected in uninfected leaves from upper part of plants infected on the lower leaves with *P. arachidicola*. Concentrations of phytoalexins in the upper leaves were 69% of the concentration in the lower leaves (table 7.4). In the lower leaves 49% of total phytoalexins was medicarpin and 34% demethylmedicarpin. In the upper leaves 43% of total phytoalexins was medicarpin and 23% demethylmedicarpin.
TABLE 7.2

Comparison of phytoalexins induced by *C. arachidicola* (C.a.) and *P. arachidicola* (P.a.)

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>TOTAL PHYTOALEXIN (µg/gram fresh weight)</th>
<th>MEDICARPIN (µg/gram fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/11/11</td>
<td>17.50</td>
<td>44.84</td>
</tr>
<tr>
<td>Egret</td>
<td>114.66</td>
<td>155.60</td>
</tr>
<tr>
<td>P84/5/244</td>
<td>25.93</td>
<td>35.80</td>
</tr>
<tr>
<td>Plover</td>
<td>99.63</td>
<td>36.89</td>
</tr>
<tr>
<td>Flamingo</td>
<td>147.97</td>
<td>34.64</td>
</tr>
<tr>
<td>MEAN</td>
<td>81.14</td>
<td>61.55</td>
</tr>
</tbody>
</table>

Correlation coefficient (r) 0.2810 0.5660

Values in the table are the mean of five replicate plots from the final sampling on 20/2/86.
TABLE 7.3

Analysis of phytoalexins in samples of healthy leaves from plants infected on the lower leaves with *C. arachidicola*

<table>
<thead>
<tr>
<th>CV</th>
<th>TOTAL PHYTOALEXIN (µg per gram fresh weight)</th>
<th>MEDICARPIN</th>
<th>DEMETHYLMEDICARPIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lower</td>
<td>upper</td>
<td>lower</td>
</tr>
<tr>
<td>A</td>
<td>17.50</td>
<td>4.7</td>
<td>4.62</td>
</tr>
<tr>
<td>B</td>
<td>114.60</td>
<td>96.79</td>
<td>53.54</td>
</tr>
<tr>
<td>C</td>
<td>25.93</td>
<td>17.28</td>
<td>6.90</td>
</tr>
<tr>
<td>D</td>
<td>147.97</td>
<td>87.31</td>
<td>51.73</td>
</tr>
<tr>
<td>E</td>
<td>99.63</td>
<td>45.05</td>
<td>26.00</td>
</tr>
<tr>
<td>MEAN</td>
<td>81.13</td>
<td>50.23</td>
<td>28.56</td>
</tr>
</tbody>
</table>

Values are the mean of five replicate plots in the final sampling on 20/2/86 of cultivars A = 6/11/11, B = Egret, C = P84/5/244, D = Flamingo and E = Plover.
### TABLE 7.4
Analysis of phytoalexins in samples of healthy leaves from plants infected on the lower leaves with *P. arachidicola*

<table>
<thead>
<tr>
<th></th>
<th>TOTAL PHYTOALEXIN</th>
<th>MEDICARPIN</th>
<th>DEMETHYLMEDICARPIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg per gram fresh weight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>lower</td>
<td>upper</td>
<td>lower</td>
</tr>
<tr>
<td>A</td>
<td>44.84</td>
<td>33.19</td>
<td>13.79</td>
</tr>
<tr>
<td>B</td>
<td>155.60</td>
<td>97.58</td>
<td>118.60</td>
</tr>
<tr>
<td>C</td>
<td>35.80</td>
<td>22.28</td>
<td>4.27</td>
</tr>
<tr>
<td>D</td>
<td>34.64</td>
<td>36.90</td>
<td>5.20</td>
</tr>
<tr>
<td>E</td>
<td>36.89</td>
<td>24.66</td>
<td>9.11</td>
</tr>
<tr>
<td>MEAN</td>
<td>61.55</td>
<td>43.06</td>
<td>30.19</td>
</tr>
</tbody>
</table>

Values are the mean of five replicate plots in the final sampling on 20/2/86 of cultivars A = 6/11/11, B = Egret, C = P84/5/244, D = Flamingo and E = Plover.
GROWING SEASON 3 - 1986/87

*Infection by C. arachidicola*

The three long season cultivars, Egret, Flamingo and P84/5/244 were susceptible to *C. arachidicola*. P84/5/244 and Flamingo were severely infected by 27/2/87 whilst Egret was only moderately infected (fig. 7.5). The mid season cultivar Swallow and the short season cultivar Plover were also moderately susceptible (fig. 7.6).

In P84/5/244, Plover and Swallow, medicarpin increased gradually as disease progressed reaching concentrations of between 10-20 μg/g.f wt. (fig 7.5a, 7.6a & b respectively).

In Flamingo medicarpin increased rapidly from mid-December as did disease severity (fig. 7.5b). However in Egret medicarpin accumulated rapidly from mid-December whereas disease progress was slow (fig. 7.5c).

*Infection by P. arachidicola*

Egret was the most susceptible cultivar to infection by *P. arachidicola* and P84/5/244 was most resistant whilst Flamingo and Plover were moderately susceptible (fig. 7.7).

Medicarpin accumulated in Flamingo and Egret at a rate similar to that of disease progression (fig. 7.7c & d). Only slight increases of medicarpin were detected in P84/5/244 and Plover (fig. 7.7a & b).
Figure 7.5 - Severity of leafspot caused by *Cercospora* (□) and medicarpin accumulation (●) in (a) P84/5/244, (b) Flamingo and (c) Egret. Points represent the mean of four replicate plots of samples collected on 1 = 20/11/86, 2 = 19/12/86, 3 = 23/1/87 and 4 = 27/2/87.
Figure 7.6 - Severity of leafspot caused by Cercospora (□) and medicarpin accumulation (■) in (a) Swallow and (b) Plover. Points are the mean of samples from four replicate plots taken on 1 = 16/12/86, 2 = 20/1/87 and 3 = 24 and 29/2/87 for swallow and Plover respectively.
Figure 7.7 - Severity of leafspot caused by *Phoma* (○) and medicarpin accumulation (■) in (a) Plover, (b) P84/5/244, (c) Flamingo and (d) Egret. Points represent the mean of samples from four replicate plots taken on 1 = 20/11/86, 2 = 19/12/86, 3 = 23/1/87 and 4 = 27/2/87 apart from Plover which was sampled at 1 = 16/12/86, 2 = 20/1/87 and 3 = 29/2/97.
Comparison of phytoalexins induced by C. arachidicola and P. arachidicola

As some samples were missing from those taken on the final sampling, data from the third sampling (second for Plover) were compared. Although there were some small differences in the amount of phytoalexins detected in Egret during infection by the two pathogens, a general comparison revealed no significant difference between total phytoalexins or medicarpin induced by either fungus (table 7.5).

Comparison of phytoalexins in healthy upper leaves of plants infected on the lower leaves with C. arachidicola

In all cultivars examined, isoflavonoid phytoalexins were detected in healthy leaves taken from the upper part of the plant when the lower parts were infected. An overall comparison of the cultivars showed that the concentration of phytoalexins in the upper leaves was 73% of that found in the lower leaves (table 7.6).

In the samples from lower leaves 55% of the total phytoalexin was medicarpin and 19% was demethylmedicarpin. In the samples from upper leaves 54% was medicarpin and 25% was demethylmedicarpin.

Comparison of phytoalexins in healthy upper leaves of plants infected on the lower leaves with P. arachidicola

In all cultivars, isoflavonoid phytoalexins were detected in the upper healthy leaves. In cultivars Egret and Flamingo the concentration of phytoalexins in the upper leaves was 35% and 47% of that found in the lower leaves respectively (table 7.7). The concentration of phytoalexins in cultivars 864/5/244 and Plover was higher in the upper leaves than that in the lower leaves.
### TABLE 7.5

Comparison of total phytoalexins and medicarpin induced by *C. arachidicola* (C.a.) and *P. arachidicola* (P.a.).

<table>
<thead>
<tr>
<th>CULTIVAR</th>
<th>TOTAL PHYTOALEXIN (μg per gram fresh weight)</th>
<th>MEDICARPIN (μg per gram fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egret</td>
<td>121.47</td>
<td>26.55</td>
</tr>
<tr>
<td>Flamingo</td>
<td>68.13</td>
<td>104.06</td>
</tr>
<tr>
<td>P84/5/244</td>
<td>10.77</td>
<td>7.63</td>
</tr>
<tr>
<td>Plover</td>
<td>42.24</td>
<td>16.67</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>60.65</td>
<td>38.72</td>
</tr>
</tbody>
</table>

**Correlation coefficient (r)**

- Total Phytoalexin: 0.2740
- Medicarpin: 0.393

All values are the mean of samples from four replicate plots taken on the third sampling of growing season 3 (1986/87).
TABLE 7.6

Analysis of total phytoalexins, medicarpin and demethylmedicarpin in healthy upper leaves of plants infected with *C. arachidicola* on the lower leaves

<table>
<thead>
<tr>
<th>CV</th>
<th>TOTAL PHYTOALEXIN</th>
<th>MEDICARPIN</th>
<th>DEMETHYLMEDICARPIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lower</td>
<td>upper</td>
<td>lower</td>
</tr>
<tr>
<td>A</td>
<td>121.47</td>
<td>77.32</td>
<td>66.29</td>
</tr>
<tr>
<td>B</td>
<td>68.13</td>
<td>36.84</td>
<td>38.26</td>
</tr>
<tr>
<td>C</td>
<td>42.24</td>
<td>34.09</td>
<td>24.17</td>
</tr>
<tr>
<td>D</td>
<td>10.77</td>
<td>9.23</td>
<td>5.30</td>
</tr>
<tr>
<td>MEAN</td>
<td>97.17</td>
<td>71.39</td>
<td>53.30</td>
</tr>
</tbody>
</table>

Values represent the mean of samples from four replicate plots for cultivars A = Egret, B = Flamingo, C = P84/5/244 and D = Plover taken on the third sampling of growing season 3.


TABLE 7.7

Analysis of total phytoalexins, medicarpin and demethylmedicarpin in healthy upper leaves of plants infected with *P. arachidicola* on the lower leaves

<table>
<thead>
<tr>
<th>CV</th>
<th>TOTAL PHYTOALEXIN (µg per gram fresh weight)</th>
<th>MEDICARPIN</th>
<th>DEMETHYLMEDICARPIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lower</td>
<td>upper</td>
<td>lower</td>
</tr>
<tr>
<td>A</td>
<td>26.55</td>
<td>9.42</td>
<td>19.09</td>
</tr>
<tr>
<td>B</td>
<td>104.66</td>
<td>48.76</td>
<td>49.84</td>
</tr>
<tr>
<td>C</td>
<td>7.63</td>
<td>9.89</td>
<td>5.72</td>
</tr>
<tr>
<td>D</td>
<td>16.67</td>
<td>40.97</td>
<td>11.34</td>
</tr>
<tr>
<td>MEAN</td>
<td>22.99</td>
<td>22.68</td>
<td>21.49</td>
</tr>
</tbody>
</table>

Values represent the mean of samples from four replicate plots for cultivars A = Egret, B = Flamingo, C = P84/5/244 and D = Plover taken on the third sampling of growing season 3 (23/1/86).
GROWING SEASON 4 - 1987/88

*Infection of Flamingo by C. arachidicola*

The long season cultivar Flamingo was moderately susceptible to *C. arachidicola*. Disease began to increase after 4/1/88 in all replicate plots (fig. 7.8).

Total phytoalexins and medicarpin accumulated rapidly from 15/1/88 at a rate correlating with increase in disease (fig. 7.8a & b).

Concentration of major phytoalexins, medicarpin, isoflavanone 1, formononetin and demethylmedicarpin increased as disease progressed. Medicarpin was the predominant phytoalexin until the end of January after which demethylmedicarpin predominated (fig. 7.9).

*Infection of Swallow by C. arachidicola*

Severity of disease increased rapidly from mid December and the plot was heavily infected by early March (fig. 7.10a).

Phytoalexins began to accumulate rapidly from mid to late January and medicarpin and demethylmedicarpin were the predominant isoflavonoids reaching concentrations of >300 μg/g.f.wt. (fig 7.10b). Isoflavanone 1 and formononetin reached concentrations of approx. 100 μg/g/f/wt.
Figure 7.8 – Severity of leafspot caused by *Cercospora X*, and accumulation of total phytoalexin (□) and medicarpin (■) in the long season cultivar, Flamingo (a) plot 10 and (b) plot 43. All points represent the mean of 14 replicates taken from each plot. Samples were collected at 1 = 3/12/87, 2 = 16/12/87, 3 = 4/1/88, 4 = 15/1/88, 5 = 29/1/88, 6 = 12/2/88 and 7 = 26/2/88.
Figure 7.9 - Concentration of major isoflavonoid phytoalexins, medicarpin (■), isoflavanone 1 (□), formononetin (X) and demethylmedicarpin (▲) in Flamingo during the 1987/88 growing season. Points represent the mean values of four replicate plots. Samples were collected at 1 = 3/12/87, 2 = 16/12/87, 3 = 4/1/88, 4 = 15/1/88, 5 = 29/1/88, 6 = 12/2/88 and 7 = 26/2/88.
Figure 7.10 - (a) Severity of leafspot caused by Cercospora (X), total phytoalexin (□) and medicarpin accumulation (■) in the mid-season cultivar Swallow.

(b) Concentration of major phytoalexins, medicarpin (■), isoflavonone 1 (□), formononetin (X) and demethylmedicarpin (▲). Points represent the mean values of 14 replicates collected at 1 = 3/12/87, 2 = 16/12/87, 3 = 6/1/88, 4 = 13/1/88, 5 = 27/1/88, 6 = 11/2/88, 7 = 24/2/88 and 8 = 9/3/88.
Disease progression and phytoalexin accumulation in short season cultivars

In JL 24, disease was quite severe when it was first assessed on 11/1/88 and by mid February it was severe (fig. 7.11a). Total phytoalexin was shown to increase rapidly as disease progressed (fig 7.11a).

Concentrations of medicarpin, isoflavanone 1 and formononetin increased gradually as disease progressed but demethylmedicarpin was the major isoflavonoid from early February when disease was severe (fig. 7.11b).

Malimba was also highly susceptible to *C. arachidicola* being severely infected by 17/2/88 and phytoalexins accumulated linearly from the end of January (fig. 7.12a).

Concentrations of medicarpin, isoflavanone 1 and formononetin increased as disease progressed but demethylmedicarpin increased rapidly from early February (fig. 7.12b).

Natal common was also highly susceptible to *C. arachidicola* and as with JL 24 and Malimba was severely infected by 17/2/88 (fig. 7.13a). Phytoalexins increased rapidly between 27/1/88 and 17/2/88 but decreased towards the end of the growing season when disease was most severe (fig. 7.13a).

Concentrations of the major phytoalexins increased as during the growing season as disease became more severe. As with the other short season cultivars, demethylmedicarpin was the predominant phytoalexin from the end of January (fig. 7.13b). Medicarpin concentrations began to decrease from early February and others decreased from mid February.
Figure 7.11 - (a) Severity of leafspot caused by *Cercospora* (X), total phytoalexin (□), medicarpin accumulation (■) in JL 24. Points represent the mean of fourteen replicates. Samples were collected at 1 = 11/1/88, 2 = 27/1/88, 3 = 17/2/88 and 4 = 8/3/88.

(b) Concentration of major phytoalexins, medicarpin (■), isoflavone (□), formononetin (X) and demethylmedicarpin (▲) in extracts from infected JL 24. Samples were collected at 1 = 17/12/87, 2 = 7/1/88, 3 = 21/1/88, 4 = 4/2/88, 5 = 18/2/88 and 6 = 8/3/88.
Figure 7.12 - (a) Severity of leafspot caused by Cercospora (X), total phytoalexin (□), medicarpin accumulation (■) in Malimba. Points represent the mean of fourteen replicates. Samples were collected at 1 = 11/1/88, 2 = 27/1/88, 3 = 17/2/88, and 4 = 8/3/88.

(b) Concentration of major phytoalexins, medicarpin (■), isoflavanone (□), formononetin (X) and demethylmedicarpin (▲) in extracts from infected Malimba. Samples were collected at 1 = 17/12/87, 2 = 7/1/88, 3 = 21/1/88, 4 = 4/2/88, 5 = 18/2/88, and 6 = 8/3/88.
Figure 7.13 - (a) Severity of leafspot caused by Cercospora (X), total phytoalexin (□), medicarpin accumulation (■) in Natal Common. Points represent the mean of fourteen replicates. Samples were collected at 1 = 11/1/88, 2 = 27/1/88, 3 = 17/2/88 and 4 = 8/3/88.

(b) Concentration of major phytoalexins, medicarpin (■), isoflavanone 1 (□), formononetin (X) and demethylmedicarpin (▲) in extracts from infected Natal common. Samples were collected at 1 = 17/12/87, 2 = 7/1/88, 3 = 21/1/88, 4 = 4/2/88, 5 = 18/2/88 and 6 = 8/3/88.
Effects of fungicides on disease progression and phytoalexin accumulation

*Cercospora* was reduced in plots of Flamingo sprayed with Vandozeb and thiophanate methyl (fig. 7.14a). However the reduction in early leafspot facilitated the progression of *Phoma arachidicola* (fig. 7.14a).

Phytoalexin concentrations were slightly higher in samples from unsprayed plots when data from samples collected on 14/2/88 (7th sampling) were compared (fig. 7.14b).

Another four plots of Flamingo were sprayed with the systemic fungicide procymidene to reduce web blotch. Application of this fungicide restricted growth of *P. arachidicola* but allowed rapid increase of *C. arachidicola* (fig. 7.15a).

There was no significant difference in concentrations of phytoalexins in samples from sprayed and unsprayed plants when data from 27/1/88 (5th sampling) was compared (fig. 7.15b).
Figure 7.14 - (a) Severity of disease caused by Cercospora arachidicola in unsprayed plots (■), and in plots sprayed with vandozeb/thiophanate methyl mixture (□). Severity of disease caused by Phoma (X) in plots sprayed with vandozeb/thiophanate methyl mixture. Samples were collected at 1 = 3/12/87, 2 = 16/12/87, 3 = 6/1/88, 4 = 13/1/88, 5 = 27/1/88, 6 = 11/2/88 and 7 = 14/2/88.

(b) Comparison of medicarpin, demethylmedicarpin and total phytoalexins in samples from control (■■■) and sprayed (■■■) plants collected on 14/2/88 (7th sampling).
Figure 7.15 - (a) Severity of disease caused by *Phoma arachidicola* in unsprayed plots (■), and in plots sprayed with procymidene (□). Severity of disease caused by *Cercospora* (X) in plots sprayed with procymidene. Samples were collected at 1 = 3/12/87, 2 = 16/12/87, 3 = 6/1/88, 4 = 13/1/88, 5 = 27/1/88, 6 = 11/2/88 and 7 = 14/2/88.

(b) Comparison of medicarpin, demethylmedicarpin and total phytoalexins in samples from control (■) and sprayed (□) plants collected on 27/1/88 (5th sampling).
DISCUSSION

In growing season 1 (1984/85) all cultivars were susceptible to early leafspot. Cultivars 6/11/11 and P84/5/244 produced only low concentrations of medicarpin (<50 µg/g.f.wt) whereas concentrations >50 µg/g.f.wt were detected in extracts from cultivars Egret and Flamingo.

The same cultivars had different responses to infection with *P. arachidicola*, 6/11/11 was highly susceptible, P84/5/244 was moderately resistant whilst Egret and Flamingo were moderately susceptible. Low concentrations of medicarpin and other phytoalexins (data not shown) were detected in P84/5/244 and 6/11/11 throughout disease progression whereas the reverse was the case in Egret and Flamingo. These results suggested that a plant's ability to produce medicarpin and indeed other isoflavonoid phytoalexins is a function of genotype. Where resistance is expressed as in cultivar P84/5/244 infected with *P. arachidicola* some other resistance mechanism must be operating.

Most cultivars accumulated similar concentrations of phytoalexins when infected with either pathogen. This was not a surprising result since both fungi are necrotrophic, causing substantial cell damage during the course of infection (Bailey, 1982).

In growing season 2 (1985/86), long season cultivars 6/11/11, Egret and P84/5/244 and short season cultivar, Plover were moderately susceptible to *C. arachidicola*. Concentrations of medicarpin increased slightly in 6/11/11 and P84/5/244 during the growing season but never exceeded 10 µg/g.f.wt. However, in Egret and Plover medicarpin increased linearly during the season reaching concentrations of 25-50 µg/g.f.wt.

When the same cultivars were infected with *P. arachidicola*, 6/11/11 and Egret were susceptible whilst Plover and P84/5/244 were moderately resistant. High concentrations of medicarpin were only found in Egret.

In all cultivars phytoalexins were detected in healthy upper leaves of plants infected on the lower leaves with either *C. arachidicola* or *P. arachidicola*. Medicarpin was the major phytoalexin
in the upper leaves although some demethylmedicarpin was detected. The apparently healthy leaves were detached and some were incubated at high humidity to confirm the absence of the pathogens (Cole, personal communication). These observations imply that the demethylmedicarpin detected in the upper healthy leaves was of plant origin, either as possible alternative intermediate to medicarpin or an intermediate to other, as yet unidentified phytoalexins or a plant degradation product (VanEtten et al., 1982).

In growing season 3 (1986/87) the long-season cultivars P84/5/244 and Flamingo were susceptible to *C. arachidicola* whereas Egret was moderately susceptible. The mid-season cultivar Swallow and short-season Plover were moderately susceptible. High concentrations of phytoalexins were detected in Egret and Flamingo from late December onwards. Less than 30 µg/g.f.wt. of medicarpin was detected in P84/5/244, Swallow and Plover.

Egret and Flamingo were susceptible to *P. arachidicola* whereas P84/5/244 was resistant and Plover was moderately susceptible. Only high concentrations of phytoalexins were detected in Flamingo and Egret.

Most cultivars produced similar concentrations of phytoalexins whether infected by *C. arachidicola* or *P. arachidicola*. However, in Egret much higher concentrations of phytoalexins were detected when infected by *C. arachidicola* even though disease levels were similar.

As in growing season 2, phytoalexins were detected in the healthy upper leaves of all cultivars regardless of pathogen present on the lower leaves. However, it was interesting to note that the cultivars resistant to *P. arachidicola*, P84/5/244 and Plover, had higher concentrations of phytoalexins in the healthy upper leaves compared to the infected lower leaves. This was not consistent with the results obtained in growing season 2 where higher concentrations of phytoalexins had been detected in the lower infected leaves. It seems unlikely, therefore, that accumulation of phytoalexins in upper leaves, would be responsible for the observed resistance.

Summarizing the main observations from the three growing seasons it is clear that all cultivars were generally consistent in their response to infection and the magnitude of phytoalexins accumulated.
Isoflavonoid phytoalexins were not responsible for resistance of some cultivars to *P. arachidica*. The detection of phytoalexins in apparently healthy upper leaves of plants infected on the lower leaves was interesting and warrants further investigation.

The slightly different approach adopted in growing season 4 (1987/88) provided a more detailed picture of phytoalexin accumulation during disease progression. Within plots there was good correlation between increase in disease and increase in phytoalexins. In all cultivars, demethylmedicarpin became the major phytoalexin as disease became severe, this was emphasized in the highly susceptible short-season cultivars, Malimba, Natal Common and JL 24 where demethylmedicarpin was the predominant phytoalexin in all but the first few samplings.

Where plots of Flamingo had been sprayed for *C. arachidica* the severity of *P. arachidica* increased and visa versa as had been recorded in previous experiments on control of early leaf spot and web blotch (Cole, 1981). Phytoalexin concentrations were similar in sprayed and unsprayed plants since they were infected by either *C. arachidica* or *P. arachidica*.

This has been the first attempt to examine the involvement of phytoalexins in host-pathogen interactions in the field, further, more detailed studies are required to understand this fully.
Chapter 8

Toxicity and Degradation of Groundnut Phytoalexins

INTRODUCTION

Work described in chapters 3, 5, 6 and 7 clearly demonstrated that *Arachis hypogaea* L. accumulates several isoflavonoid phytoalexins during infection by leaf spot pathogens. Although the toxicity of many of these compounds has been investigated (Adesanya *et al.*, 1966; Skipp and Bailey, 1977) it was necessary to obtain some information on toxicity towards the groundnut pathogens. None of these compounds had been tested on groundnut pathogens.

There are several methods by which to assay the toxicity of isolated phytoalexins and no one method alone provides adequate information on toxicity. Methods commonly used include germination inhibition assays, germtube elongation, colony growth on solid agar and growth in liquid media (Bailey *et al.*, 1976; Skipp and Bailey, 1977).

Ideally a combination of two or more methods is desirable but compound availability and stability are often limiting factors.

Since medicarpin was generally the dominant phytoalexin its toxicity to the foliar pathogens, *Cercospora arachidicola* and *Phoma arachidicola*, was examined in growth experiments where the fungi were grown in liquid media supplemented with a range of concentrations of medicarpin. The non-pathogen *Cladosporium cucumerinum* was also examined.

A general indication of the toxicities of other compounds was obtained by investigating inhibition of spore germination from a selection of pathogens and non-pathogens.

A large number of fungal pathogens have been demonstrated to be tolerant of their hosts phytoalexins and in fact a large number have been shown to modify and degrade the compounds (VanEtten *et al.*, 1982, 1989), thus enabling their survival in plant tissues capable of
accumulating high concentrations of phytoalexins. Much work has been done on the ability of *Nectria haematococca*, a pea pathogen, to degrade the major phytoalexin of pea, pisatin, by removal of the 3-O-methyl group (VanEtten et al., 1975). Conventional genetical analysis and transformation studies have shown phytoalexin detoxification is an essential requirement for pathogenicity (Kistler and VanEtten, 1984ab; Tegtmeier and VanEtten, 1982). Avirulent isolates were unable to degrade pisatin and were sensitive to the phytoalexin.

Many host-pathogen systems have been investigated and there appear to be several alternative mechanisms of primary modification which include oxidation, hydration and carbonyl reduction or ether cleavage (VanEtten et al., 1982). Enzymes catalysing some of these reactions in particular systems have been isolated and partially characterized such as pisatin demethylase, a microsomal cytochrome P-450 monooxygenase from *N. haematococca* (Matthews and VanEtten, 1983) and the extracellular kievitone hydratase from *Fusarium solani* f.sp. *phaseoli* (Kuhn and Smith, 1979) which catalyses hydration of the isopentenyl side chain of the isoflavonoid, kievitone.

Biochemical and genetic studies have shown that the ability of pathogens to detoxify their host's phytoalexins may be important in pathogenesis although there are many cases where this clearly is not a requirement. Tolerance is not always associated with the ability to metabolize phytoalexins so it seems important to examine each host-pathogen system individually to determine the importance of such mechanisms. A well studied example concerns the aggressive pea pathogen, *Aphanomyces euteiches*, which is sensitive to pisatin *in vitro* but elicits high concentrations of pisatin during infection (Pueppke and VanEtten, 1976). The fungus apparently grew in contact with the high concentrations of pisatin but to date there has been no suggestion as to the mechanism of tolerance (Sweigard and VanEtten, 1987).

Many fungi have the ability to metabolize medicarpin by at least one route to give generally more polar products which are less toxic (fig. 8.1). These reactions include oxidative formation of the 1α-hydroxy dienone (reaction A; Denny and VanEtten, 1982), 9-O demethylation as already mentioned (reaction B; Weltring and Barz,
1980; Kraft and Barz, 1985; Kraft et al., 1987), reductive cleavage of ring C to give the isoflavan, vestitol (reaction C; Weltring et al., 1981; VanEtten et al., 1982), oxidative opening of dihydrofuran ring giving vestitone (reaction D; Denny and VanEtten, 1982) and C-6a and C-4 hydroxylation (reactions E and F respectively; Denny and VanEtten, 1982; Ingham, 1976). In some cases the fungi are able to metabolize these products further but the nature of the experimental conditions may have some effect on this depending on what carbon sources are available.

Assessment of phytoalexin accumulation in Arachis hypogaea infected with foliar pathogens in the greenhouse and in the field indicated that several isoflavonoids may in actual fact be degradation products. Since it had not been possible to identify the phytoalexins produced only by the plant by use of abiotic elicitors, it was necessary to examine the response of the pathogens, growing in culture, to medicarpin.

Degradation products were investigated by several chromatographic-spectroscopic techniques including GC-MS, LC-MS and reverse phase HPLC with diode array detection which had been used successfully to identify isoflavonoids in extracts from infected groundnut foliage as described in chapter 3. Where possible authentic material was obtained for comparison.
Figure 8.1 - Primary modifications of medicarpin by fungi.

Reaction A - oxidative conversion to 1α-hydroxy-9-methoxy-pterocarp-1(2), 4-diene-3-one.

Reaction B - 9-O-demethylation giving demethylmedicarpin (3,9-dihydroxypterocarpan).

Reaction C - reductive cleavage of benzylphenylether bond to give vestitol (7,2′dihydroxy-4′methoxy-isoflavan).

Reaction D - Oxidative opening of dihydrofuran ring to give vestitone (7,2′dihydroxy-4′methoxy isoflavanone).

Reaction E and F - Hydroxylation at C-6a and C-4 respectively.
MATERIALS AND METHODS

Chemicals

Phytoalexins medicarpin, formononetin, demethylmedicarpin and isoflavanone 1 (7,4'-dimethoxy-2'-hydroxyisoflavanone) were isolated as described in chapter 3.

Fungi

Organisms tested were the pathogens, Cercospora arachidicola and Verticillium dahliae and the non-pathogens were Cladosporium cucumerinum, Fusarium moniliformin and Curvularia lunata. All cultures were maintained on potato dextrose agar (PDA; Oxoid, Basingstoke U.K.) apart from C. arachidicola which was maintained on peanut oatmeal agar (Smith, 1971). Spores were harvested in Czapek Dox liquid medium by agitation with a glass rod. The resulting suspension was passed through four layers of muslin to remove mycelial debris and the final suspension was adjusted to 10^6 spores ml^-1.

Phoma arachidicola was isolated from infected groundnut leaves in Zimbabwe as described in chapter 5 and was maintained on PDA.

Germination inhibition

Phytoalexins were taken up in ethanol and applied as 5 μl droplets to multiwell microscope slides (Flow Laboratories, Irvine, U.K.) and allowed to dry. Spore suspensions (5 μl) were placed in the wells giving final concentrations of phytoalexins of 0, 10, 20, 40, 80 and 160 μg/ml. The slides were incubated in dark moist conditions for 18 h. The wells were examined by light microscopy and 100 spores in each well were counted for germination. Spores with germ tubes longer than the diameter of the spore were scored as germinated. Each treatment was done in duplicate wells and the mean of the two was used for subsequent calculations. The percentage inhibition caused by the phytoalexins was calculated using the formula \((C-T)/C\) x 100, where C = mean percentage germination in controls and T = mean percentage germination in tests. Percentage inhibition was converted to probit values (Finney, 1952) and plotted against \(\log_{10}\) phytoalexin concentration to obtain the ED_{50}. 

-179-
Growth Inhibition

Medicarpin (41 mg) was obtained by the chromatographic methods described in chapter 3.

*Cercospora arachidicola*, *Phoma arachidicola* and *Cladosporium cucumerinum* were subcultured by removing 4 x 4 mm disks, using a cork borer, from cultures on agar plates where mycelial coverage was uniform, into Czapek Dox liquid medium (5 ml) in 100 ml Erlenmeyer flasks. Since both these fungi grew slowly, especially *Cercospora*, it was not possible to do this experiment by removing quantified amounts of mycelia as would have been desirable.

After inoculation, the fungi were allowed to grow for 24 hours. Medicarpin was filter sterilized using a 0.2 μm filter (Millipore) and added to the flasks at concentrations of 0, 10, 20, 40 and 80 μg/ml and three batch cultures for each sample were set up. The *Cercospora* and *Phoma* were incubated at 28°C in the light and the *Cladosporium* was incubated at 26°C in the dark. All cultures were agitated daily.

Samples (three replicates) for each concentration were removed at 2 days, 4 days, 8 days and 16 days apart from *Cercospora* which was sampled at 4, 8 and 16 days after the addition of medicarpin.

Media were vacuum filtered through a preweighed GFC filter (Whatman) and wet weights of fungal cells were obtained. Filters were dried at 60°C for 24 h before weighing to obtain dry weights of fungal material. Filtrates were retained for separate analysis.

Investigation of medicarpin degradation

Filtrates retained from the growth experiment were extracted twice with two volumes of ethyl acetate. Ethyl acetate fractions were pooled and dried over anhydrous sodium sulphate. The solvent was then removed by rotary evaporation and the residue was taken up in 1 ml of acetonitrile for further analysis.

A short time course experiment was set up to determine if medicarpin was degraded by *C. arachidicola* and *P. arachidicola* over 26 hours. Samples were inoculated as described for the longer growth experiment and 24 hours after inoculation, 20 μg/ml of filter sterilized medicarpin was added. Samples (3 replicates) were extracted at intervals over 26 h. after addition of medicarpin.
**Analytical HPLC**

Medicarpin and its degradation products were separated by reverse phase HPLC as described earlier but with a modified gradient to account for the simplicity of the samples. The solvents were as before, solvent A was 1% acetic acid and solvent B was acetonitrile. The binary gradient was 40% B increasing to 60% B over 12 minutes. Quantification was by external standardisation as described in chapter 4 and major unknown degradation products were quantified using the response factor obtained for medicarpin as the compounds had similar U.V. absorbance characteristics.

**Identification**

Compounds were identified on the basis of retention time and UV spectra as described in chapter 3.

**LC-MS**

Once run on analytical HPLC with diode array detection, a selection of samples were run on an alternative system with mass detection. Components in the samples were separated on a Hichrom ODS 2 column (150 mm × 4.9 mm i.d.; 3 μm particle size). Solvent A was 20% CH$_3$CN/aq. 0.1 M NH$_4$OAc and B was 80% CH$_3$CN/aq. 0.1 M NH$_4$OAc. A binary gradient starting at 30% B increasing to 70% B over 12 min. The mobile phase was maintained at 70% B for 3 min. and equilibrated to 30% B over 2 min. Components were monitored by positive ion discharge. Other operational parameters were as described in chapter 3.

**GC-MS**

Aliquots of samples extracted as described were freeze dried and taken up in 250 μl of methanol. 50 μl of this was removed and dried in a reaction vial. Phenolics were derivatized in a mixture of trimethylchlorosilane (Sigma) and pyridine (1:1).

A Carlo Erba Strumentazione HPGC 5160 was fitted with a BP1 fused silica quartz capillary column (50 m with 0.5 μm film; SGE). Helium was the carrier gas with a column head pressure of 130 KPa.
Detection was by a Hewlett Packard 5970 series mass selective detector. Electron ionisation energy was 70 ev.

Components were separated on the following GC program: 50°C increasing to 250°C at 39.9°C/min, with a further increase to 300°C at a rate of 5°C/min where it was maintained for a further 15 min.
RESULTS

Germination Inhibition

Percentage germination was converted to probit values (Finney 1952) and plotted against log₁₀ phytoalexin concentration to enable calculation of the ED₅₀ value. Results showed that all organisms were sensitive to medicarpin and isoflavanone 1 with ED₅₀ values between 5 and 20 µg/ml (table 8.1).

Table 8.1
Inhibition of spore germination of five fungi by four phytoalexins.

<table>
<thead>
<tr>
<th>FUNGI</th>
<th>Concentration of phytoalexin (µg/ml) required to give 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MED</td>
</tr>
<tr>
<td>Cercospora arachidicola</td>
<td>5</td>
</tr>
<tr>
<td>Cladosporium cucumerinum</td>
<td>17</td>
</tr>
<tr>
<td>Fusarium moniliforme</td>
<td>7</td>
</tr>
<tr>
<td>Curvularia lunata</td>
<td>20</td>
</tr>
<tr>
<td>Verticillium dahliae</td>
<td>10</td>
</tr>
</tbody>
</table>

N = not tested
MED = medicarpin, INE 1 = 7,4'-dimethoxy-2'-hydroxyisoflavanone, FORM = formononetin and DEM = demethylmedicarpin.
Isoflavanone 1 was almost as inhibitory as medicarpin to *C. arachidicola* and the non-pathogens of groundnut whereas formononetin and demethylmedicarpin inhibited germination only at high concentrations. Demethylmedicarpin was considerably less inhibitory than medicarpin.

**Growth Inhibition**

As medicarpin was the most abundant and inhibitory phytoalexin, its effect on growth of fungi in liquid cultures was examined. Satisfactory correlation was obtained between dry and wet weight hence only dry weights are discussed below.

Cultures of *C. arachidicola* with media supplemented with 0 to 80 µg/ml showed little or no variation in growth over the 16 day period of the experiment (fig. 8.2). Since the fungus was so slow growing it was impossible to assess if the medicarpin was having any effect.

Growth of *P. arachidicola* in cultures supplemented with lower concentrations of medicarpin (10 and 20 µg/ml) was not significantly different from the control (fig. 8.3). In contrast, the higher concentration of 80 µg/ml was seen to have a fungistatic affect initially but growth did pick up and increase after 16 days. The intermediate concentration of 40 µg/ml was shown to cause a drop in dry weight initially (possibly due to a leakage of electrolytes often associated with phytoalexin toxicity) but the fungus had doubled in dry weight by 8 days.

*Cladosporium*, a pathogen to cucumbers, was used to examine the effect of medicarpin on a non-pathogen of groundnuts. Medicarpin concentrations >20 µg/ml resulted in a decrease in dry weight whereas the control and the culture containing 10 µg/ml increased up to 8 days after addition of medicarpin (fig. 8.4)
Figure 8.2 - Effect of different concentrations of medicarpin, 0 µg/ml ( ■ ), 10 µg/ml ( □ ), 20 µg/ml ( + ), 40 µg/ml ( ▲ ) and 80 µg/ml ( X ), on the growth of C. arachidicola as assessed by dry weight. Batch cultures with 3 replicates for each treatment were used and the mean values are represented above. Standard deviations ranged between 5 and 35%.
Figure 8.3 - Effect of different concentrations of medicarpin, 0 µg/ml (■), 10 µg/ml (□), 20 µg/ml (+), 40 µg/ml (▲) and 80 µg/ml (X), on the growth of *P. arachidicola* as assessed by dry weight. Points represent the mean values of three replicate batch cultures.
Figure 8.4 - Effect of different concentrations of medicarpin, 0 µg/ml (■), 10 µg/ml (□), 20 µg/ml (+), 40 µg/ml (▲) and 80 µg/ml (X), on the growth of C. cucumerinum as assessed by dry weight. Batch cultures with three replicates for each treatment were used.
Metabolism of medicarpin

The media from the growth inhibition experiments was extracted and analysed for the presence of medicarpin and both the two pathogens and the non-pathogen were shown to metabolize medicarpin.

Metabolism of medicarpin by C. arachidicola

Analytical HPLC

Extracts from media, four days after addition of medicarpin, were shown to contain the more polar pterocarpan 3,9 dihydroxypterocarpan otherwise known as demethylmedicarpin. This compound was identified by comparison of retention time and UV spectra of unknown to standard, both having absorbance maxima at 285 nm (fig. 8.5 and 8.6).

LC-MS

For additional confirmation, some samples which had been examined by analytical HPLC were run on an alternative reverse phase HPLC system with mass detection. The major component was not medicarpin but demethylmedicarpin with MH⁺ of 257, consistent with a mass of 256 for this compound (fig. 8.7). Another, more polar ion, than medicarpin was detected with MH⁺ of 181. Medicarpin was not present.

GC-MS

Aliquots of samples analysed by LC-UV were derivatised and the resulting TMS ether derivatives were separated by gas chromatography with mass detection. Demethylmedicarpin was a major component as identified by comparison to authentic material (table 8.2). Little or no medicarpin remained in the samples.
TABLE 8.2

Identification of demethylmedicarpin in samples extracted from *C. arachidicola* culture media supplemented with medicarpin by comparison of retention time and fragmentation pattern of TMS derivatives with those of authentic material.

<table>
<thead>
<tr>
<th>Major ions [m/z (%)]</th>
<th>STANDARD</th>
<th>UNKNOWN</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET. TIME</td>
<td>24.64 min.</td>
<td>24.60 min.</td>
</tr>
<tr>
<td>401 (35.26)</td>
<td>401 (35.36)</td>
<td></td>
</tr>
<tr>
<td>400 (100)*</td>
<td>400 (100)</td>
<td></td>
</tr>
<tr>
<td>385 (9.20)</td>
<td>385 (9.39)</td>
<td></td>
</tr>
<tr>
<td>219 (10.43)</td>
<td>219 (11.74)</td>
<td></td>
</tr>
<tr>
<td>207 (7.00)</td>
<td>207 (6.70)</td>
<td></td>
</tr>
<tr>
<td>206 (13.60)</td>
<td>206 (13.39)</td>
<td></td>
</tr>
<tr>
<td>191 (4.41)</td>
<td>191 (13.12)</td>
<td></td>
</tr>
<tr>
<td>185 (6.17)</td>
<td>185 (5.39)</td>
<td></td>
</tr>
</tbody>
</table>

* Base peak on which percentage abundance was calculated
Figure 8.5 - Separation of standard isoflavonoid phytoalexins on a short gradient running from 40% to 60% acetonitrile over 12 min. A 10 μl sample containing 1, daidzein; 2, demethylmedicarpin; 3, isoflavanone 2 (7,2'-dihydroxy-4'-methoxyisoflavanone); 4, formononetin; 5, isoflavanone 1 (7,4', dimethoxy-2'-hydroxyisoflavanone); 6, medicarpin.
Figure 8.6 - Chromatogram of extract of media from a culture of *Cercospora arachidicola* 4 days after the addition of medicarpin (80 μg/ml). The insert shows the UV spectra obtained from the peak 2 (demethylmedicarpin) eluting at 5.18 min. At the beginning of the experiment there was only one medicarpin peak (6).
Figure 8.7 - LC-MS of an extract of media from a culture of *Cercospora arachidicola* 4 days after addition of medicarpin (80 µg/ml). Two peaks were detected as shown by the total ion chromatogram (top). A major peak at $t_r$ 4.78 min. had a mass spectrum with a prominent ion of $M^+ \text{ of } 257$ consistent with demethylmedicarpin (centre). A mass spectrum at $t_r$ 9.94 min. (bottom) shows an ion of $M^+ \text{ of } 181$. 
Time course of metabolism of medicarpin by Cercospora arachidicola

Medicarpin and demethylmedicarpin concentrations were analysed in culture filtrates retained from the 16 day growth inhibition experiment. Only trace amounts of medicarpin were detected in media from Cercospora cultures four days after medicarpin had been added (fig. 8.8) despite the small amount of fungus present. On the other hand concentrations of demethylmedicarpin were shown to reach a maxima after 4 days (fig. 8.9). After 4 days concentrations of demethylmedicarpin began to decline. Other compounds must have been formed since if demethylmedicarpin was the only major product, concentrations of this plus remaining medicarpin would have been similar to the initial concentration of medicarpin. No other isoflavonoids were detected in samples examined after 16 days growth.

A similar experiment was carried out but samples were analysed over a 26 hour period. Within the first ten hours concentrations of medicarpin began to decrease and concentrations of demethylmedicarpin began to increase. By 26 hours the isoflavonoid composition of the media consisted of approximately 50% of each pterocarpan. (fig. 8.10)
Figure 8.8 - Assessment of medicarpin remaining in cultures of *C. arachidicola* where the medium was supplemented with a range of concentrations; 10 μg/ml (■), 20 μg/ml (□), 40 μg/ml (+) and 80 μg/ml (▲).
Figure 8.9 - Assessment of demethylmedicarpin in cultures of C. arachidicola which had been supplemented with a range of concentrations of medicarpin; 10 µg/ml (■), 20 µg/ml (□), 40 µg/ml (+) and 80 µg/ml (▲).
Figure 8.10 - Assessment of demethylmedicarpin (□) and medicarpin (■) concentrations in cultures of C. arachidicola where the media had been supplemented with 100 µg of medicarpin.
Metabolism of medicarpin by P. arachidicola

LC-UV

Media extracted from cultures of *P. arachidicola* supplemented with medicarpin contained several compounds that were more hydrophilic than medicarpin. The major component in most samples had a similar retention time and UV absorption spectrum to that of 7-2'-dihydroxy-4'methoxy isoflavanone (fig. 8.11). Co-chromatography was also obtained on the longer gradient used for analysis of phytoalexins in groundnut leaf extracts. Traces of demethylmedicarpin were also identified on the basis of similar retention time and UV spectra.

LC-MS

Medicarpin was the major component 2 days after addition of higher concentrations of medicarpin (80 µg/ml) to the culture (fig. 8.12). Several components, more hydrophilic than medicarpin, were detected. A peak at retention time 4.16 min. contained several ions of m/z 257, 276 and 289 suggesting coelution (fig. 8.12). An ion chromatogram at m/z 287 revealed three peaks.

GC-MS

In all the samples examined the trimethylsilyl derivative of medicarpin (m/z 342 amu) was detected but no other isoflavonoids were identified (results not shown). This may have been due to the long delay between experiment and analysis.
Figure 8.11 - Chromatogram of extract of media from a culture of *Phoma arachidicola* 2 days after addition of medicarpin (80 μg/ml). Medicarpin was still present but several peaks had appeared and the one at retention time 5.80 min. (peak 3) had a spectrum characteristic of an isopflavanone (insert). Demethylmedicarpin was also detected (peak 2) by comparison of retention time and UV spectra to those of authentic material.
Figure 8.12 - Total ion chromatogram (a) of media from a culture of *Phoma arachidicola* 2 days after addition of medicarpin (80 µg/ml). A major peak at *t*ₘ 12.17 min. had MH⁺ 271 consistent with that obtained for medicarpin (b). A peak at *t*ₘ 4.16 min. was composed of several ions (c). An ion chromatogram at m/z 287 revealed three peaks (d).
Metabolism of medicarpin by Phoma arachidicola

Analysis of media in the growth inhibition experiment showed that medicarpin decreased almost linearly with time (fig. 8.13) and that at the lower concentrations, 10-40 μg/ml media, had been completely metabolized by the 4th day.

The isoflavanone accumulated to maximum concentrations by the 2nd day (fig. 8.14) in cultures supplemented with concentrations of 10, 20 and 40 μg/ml but although there was an obvious accumulation of this metabolite in the culture supplemented with the higher concentration by the 2nd day it continued to increase reaching a maximum at day 8.

As with *C. arachidicola*, medicarpin degradation by *P. arachidicola* was examined over a 26 hour period. A similar pattern was obtained to that with *C. arachidicola*, medicarpin concentration began to decrease after about 6 hours with a concomitant increase in the isoflavanone metabolite (fig. 8.15)
Figure 8.13 - Analysis of medicarpin in media supplemented with a range of concentrations of medicarpin, 10 µg/ml (■), 20 µg/ml (□), 40 µg/ml (+) and 80 µg/ml (▲), during growth of *Phoma arachidicola*. 
Figure 8.14 - Analysis of major metabolite, possibly an isoflavanone, in culture media from *P. arachidicola* where the media had been supplemented with a range of concentrations of medicarpin, 10 µg/ml (■), 20 µg/ml (□), 40 µg/ml (+) and 80 µg/ml (▲).
Figure 8.15 - Assessment of medicarpin (■) and isoflavanone (□) concentrations in media of *F. arachidicola* which had been supplemented with 100 µg of medicarpin.
Metabolism of medicarpin by C. cucumerinum

Analytical HPLC

This fungus was shown to form another class of metabolite. As with the other fungi the major product of medicarpin metabolism was more polar, having a retention time of 5.08 min. The UV spectrum of this peak had a λ max of 286 nm and a shoulder 310 nm (fig. 8.16).

LC-MS

In cultures examined 2 days after addition of medicarpin (40 µg/ml) major compounds were medicarpin, as indicated by m/z 271 and two compounds with m/z 285 at retention times 5.28 and 2.93 min. (fig. 8.17).

GC-MS

No useful data was obtained from these analyses.

Time course of medicarpin metabolism in liquid culture

The lower concentrations of medicarpin, 10-40 µg/ml had been removed by the 4th day, but the high concentration of 80 µg/ml disappeared more slowly and traces were still detectable after 8 days (fig. 8.18). In the same media the major metabolite accumulated rapidly over the first 2 days in cultures supplemented with all concentrations of medicarpin before decreasing (fig. 8.19). This metabolite was only detected in the media that had been supplemented with the higher concentrations of medicarpin by 16 days.
Figure 8.16 - Chromatogram of media extract from a culture of *C. cucumerinum* 2 days after addition of medicarpin (40 μg/ml). Some medicarpin was detectable (peak 6) but a major peak had absorbance maxima at 286 nm (insert).
Figure 8.17 - Total ion chromatogram of media extract from a culture of *C. cucumerinum* 2 days after addition of medicarpin (40 µg/ml) (a). A mass spectrum of the peak at 11.05 min. consisted of ions m/z 271 indicative of medicarpin (b). Mass spectra selected from the peaks at t<sub>r</sub> 5.28 and 2.93 min contained prominent ions at m/z 285, the latter also containing a major ion at m/z 181 (c and d).
Figure 8.18 - Analysis of medicarpin concentration in media supplemented with 10 µg/ml (■), 20 µg/ml (□), 40 µg/ml (+) and 80 µg/ml (▲) of medicarpin during growth of C. cucumerinum.
Figure 8.19 - Analysis of major metabolite, polar compound with absorption maxima of 286 nm, in media supplemented with 10 µg/ml (■), 20 µg/ml (□), 40 µg/ml (+) and 80 µg/ml (▲) of medicarpin during growth of C. cucumerinum.
DISCUSSION

Medicarpin and isoflavanone 1 inhibited spore germination of both pathogens and non-pathogens at concentrations between 5-20 µg/ml⁻¹ whereas formononetin and demethylmedicarpin were only effective at concentrations >80 µg/ml⁻¹. However, growth of the pathogens, *C. arachidicola* and *P. arachidicola* in media supplemented with different concentrations of medicarpin was not inhibited at concentrations of 40 µg/ml⁻¹ or less and at higher concentrations growth proceeded at a slower rate but it was not completely inhibited. In contrast in the non-pathogen of groundnut, *C. cucumerinum*, decreases in dry weights were observed when the media was supplemented with concentrations of medicarpin in excess of 20 µg/ml. These observations were consistent with those of other workers in that ageing fungi are less sensitive to phytoalexins than spores and sporelings (Skipp and Bailey, 1977; Stoessl, 1983). These results illustrate the requirement for more than one toxicity bioassay and a need for cautious interpretation of results.

Analysis of medicarpin in media during growth inhibition assays showed a rapid decrease in concentration with an increase in more polar compounds in all fungi tested. The major component in media inoculated with *C. arachidicola* was demethylmedicarpin. O-demethylation at C9 is also performed by *Aschochyta rabiei* (Kraft et al., 1987) and *Fusarium* spp. (Weltring et al., 1981). This finding supports earlier results on the accumulation of demethylmedicarpin in infected leaf tissue as discussed in chapters 5, 6 and 7.

In cultures of *P. arachidicola* the major metabolite was shown to be the isoflavanone, vestitone, by cochromatography and comparison of UV spectra at similar retention times. Vestitone had been reported as a product of fungal metabolism of medicarpin by oxidative opening of the dihydrofuran ring (Denny and VanEtten, 1982). Demethylmedicarpin was also identified in the media from some samples. Compounds with molecular weights corresponding to demethylmedicarpin and a dihydroxy, monomethoxy isoflavanone were detected by LC-MS. However these results were not confirmed by GC-MS. Since these analyses were
performed at least six months after the experiments it is possible that the compounds were no longer detectable due to instability. More experiments are required for a more precise identification of metabolites produced by *P. arachidicola*.

*C. cucumerinum* was also shown to metabolize medicarpin *in vitro* with increases in a more polar compound which had a UV spectrum similar to a 1a-hydroxydienone. However further work is required to confirm this. Although *C. cucumerinum* is not a pathogen of groundnut or other hosts producing medicarpin, it is not unusual for a non-pathogen to be able to detoxify phytoalexins that it would not normally encounter.

In the longer time course experiment where growth and phytoalexins were monitored no traces of medicarpin or any of the primary metabolites were detectable by the end of the experiment indicating complete metabolism. To confirm if this were the case it would be useful to use radiolabelled medicarpin to determine if carbon dioxide was the end product of metabolism. It would also be necessary to examine the production of other intermediates of medicarpin catabolism which would not be extracted by procedures used in the present study i.e. extraction into ethyl acetate will not extract highly polar components from the media.
CONCLUSION

The identification of common isoflavonoid phytoalexins in groundnut leaves infected with fungi is similar to many other members of the Leguminosae (Ingham, 1982). In other systems the biosynthetic pathway leading to medicarpin has been elucidated using several biochemical approaches (Dewick, 1982; Woodward, 1981; Barz et al., 1988). In such systems intermediates included daidzein, formononetin and vestitone. Since these compounds were found accompanying medicarpin in extracts from infected groundnut tissues it is most probable that these are precursors of medicarpin in the groundnut. The partial characterization of 7,4'-dimethoxy-2'-hydroxy isoflavanone may indicate the presence of a branch in this biosynthetic route rather than an alternative route, since this compound was always accompanied by medicarpin.

Results from infection studies throughout the study indicated that demethylmedicarpin was a product of fungal metabolism of medicarpin. However, demethylmedicarpin was often detected in the healthy upper leaves of plants infected on the lower leaves with either C. arachidicola or P. arachidicola. This may suggest that this compound may be synthesised by the plant, especially since 7,2',4'trihydroxyisoflavanone, a known precursor, was identified in infected leaves. On the other hand, both of these compounds could be formed as a result of the plant's own metabolism (VanEtten et al., 1982). The isoflavonoids characterized or partially characterized are summarized in appendix B (page 215-216).

In vitro experiments with C. arachidicola and P. arachidicola supported the view that the majority of the demethylmedicarpin detected was due to fungal metabolism. These pathogens, analogous to others where the host produces medicarpin, were able to demethylate the medicarpin to produce less toxic compounds (VanEtten, 1989).

Experiments using chemicals as elicitors resulted in production of low concentrations of medicarpin compared with concentrations detected during infection of certain cultivars. The implication of these results is that a specific elicitation response occurs during
pathogenesis resulting in accumulation of high concentrations of phytoalexins. There may be a fungal component, common to the leafspot pathogens investigated, which acts as an elicitor such as polysaccharides, proteins, glycoproteins and unsaturated fatty acids which have been isolated from other pathogens (Darvill and Albersheim, 1984). Alternatively, endogenous elicitors, such as oligo-α-1,4-D-galacturonide, liberated by plant hydrolases upon wounding of plant tissue or by the action of fungal polygalacturonases, may be involved (Darvill and Albersheim, 1984; de Wit, 1986). It was recently shown that some plants are able to regulate the fungal endopolygalacturonases which normally degrade polygalacturonides into oligomers which are too short to possess elicitor activity. A constitutive plant protein prolongs the half-life of the of the elicitor active polygalacturonides resulting in a stronger defence response (Cervone et al., 1989). These reports illustrate the importance of the endogenous elicitor in complex host-pathogen interactions. It would be interesting to determine elicitor active moieties in groundnut leaves and several leafspot pathogens. Are there pathogen specific elicitors? Or is the process of elicitation similar whether the leaves are infected by *P. arachidis* or *C. arachidicola*?

The present study has shown that phytoalexins alone are not responsible for resistance but that in some cultivars, which have the ability to produce high concentrations of phytoalexins rapidly, this may impart some tolerance since the fungus has to deal with it. In the greenhouse experiments Egret was less susceptible and produced higher concentrations of the more fungitoxic phytoalexins than P84/5/244 suggesting that the higher concentrations of these compounds retarded the progress of the pathogens. In order to obtain a clear understanding of resistance of groundnuts to leafspot pathogens further investigations of other defence mechanisms as well as pathogenicity factors are required. The use of resistant cultivars and wild *Arachis* species which have demonstrated immunity to some leafspot pathogens would be essential for these investigations.

The main conclusions from this preliminary investigation into the involvement of phytoalexins in resistance of groundnuts to foliar pathogens can be summarised:
(1) Mechanisms of phytoalexin elicitation are specific
(2) Rapid accumulation of high concentrations of phytoalexins in some
cultivars may retard the fungus.
(3) Many pathogens and non-pathogens have the ability to detoxify
medicarpin, the most toxic phytoalexin produced.
(4) In most highly susceptible cultivars, demethylmedicarpin was the
predominant compound.
### Appendix A

#### TABLE A

Retention times of isoflavonoid phytoalexins relative to medicarpin in two reverse phase HPLC systems.

<table>
<thead>
<tr>
<th>CMPD.</th>
<th>$t_R$ system 1&lt;sup&gt;a&lt;/sup&gt; (relative to medicarpin)</th>
<th>$t_R$ system 2&lt;sup&gt;b&lt;/sup&gt; (relative to medicarpin)</th>
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<tbody>
<tr>
<td>Medicarpin</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Isoflavanone 1</td>
<td>0.95</td>
<td>0.94</td>
</tr>
<tr>
<td>Formononetin</td>
<td>0.91</td>
<td>0.89</td>
</tr>
<tr>
<td>Isoflavanone 2</td>
<td>0.63</td>
<td>0.61</td>
</tr>
<tr>
<td>Demethylmedicarpin</td>
<td>0.53</td>
<td>0.51</td>
</tr>
<tr>
<td>Isoflavanone 3</td>
<td>0.47</td>
<td>0.48</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.43</td>
<td>0.39</td>
</tr>
</tbody>
</table>

<sup>a</sup> In system 1, compounds were separated on a column of ODS 1 (250 x 4.6 mm i.d.; 10 µm particle size) with a mobile phase consisting of a gradient of acetonitrile in 1% acetic acid in which the acetonitrile concentration was increased from 35% to 40% over the first 12 min and then to 75% over the next 23 min. A reequilibration time of 5 min was allowed to elapse before the next sample was injected.

<sup>b</sup> In system 2 compounds were separated on a column of ODS 2 (150 x 4.6 mm i.d.; 3 µm particle size). The mobile phase consisted (A) 20% acetonitrile in 0.1 M ammonium acetate and (B) 80% acetonitrile in 0.1 M ammonium acetate. Solvent B was maintained at 15% for 1 min, increased to 25% over the next 12 min and to 90% over the following 18 min. The column was reequilibrated by decreasing B over the next 4 min and maintaining this for a further 3 min.
Appendix B

<table>
<thead>
<tr>
<th>STRUCTURE</th>
<th>NAME (TOxicity*)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Medicarpin" /></td>
<td>Medicarpin (5-20 µg/ml) 270 mwt. produced by leaves during infection with foliar pathogens or by treatment with abiotic elicitors</td>
</tr>
<tr>
<td><img src="image2" alt="Formononetin" /></td>
<td>Formononetin (&gt;160 µg/ml) 268 mwt. produced by leaves during infection with foliar pathogens along with medicarpin. Most likely a precursor of medicarpin.</td>
</tr>
<tr>
<td><img src="image3" alt="Isoflavanone 1" /></td>
<td>Isoflavanone 1 (16-30 µg/ml) (7,4'-dimethoxy-2'hydroxyisoflavanone) 300 mwt. Detected in infected leaves along with medicarpin and formononetin</td>
</tr>
<tr>
<td><img src="image4" alt="Isoflavanone 2" /></td>
<td>Isoflavanone 2 (Vestitone) (7,2'-dihydroxy-4'methoxyisoflavanone) 286 mwt. metabolite of medicarpin by P. arachidicola possible precursor of medicarpin</td>
</tr>
</tbody>
</table>

contd...
**TABLE B contd.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>M.W.T.</th>
<th>Description</th>
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<tbody>
<tr>
<td>Isoflavanone 3</td>
<td><img src="image" alt="Isoflavanone 3" /></td>
<td>286 mwt</td>
<td>(dihydroxy-monomethoxyisoflavanone) minor isoflavanone could be produced by the leaves and/or be a product of fungal modification of medicarpin</td>
</tr>
<tr>
<td>Demethylmedicarpin (&gt;90 µg/ml)</td>
<td><img src="image" alt="Demethylmedicarpin" /></td>
<td>256 mwt</td>
<td>major product of medicarpin metabolism by <strong>C. arachidicola</strong> and <strong>P. arachidicola</strong> a minor concentration may be produced by the plant</td>
</tr>
<tr>
<td>Daidzein</td>
<td><img src="image" alt="Daidzein" /></td>
<td>254 mwt</td>
<td>possible precursor of medicarpin</td>
</tr>
<tr>
<td>(±)-2'-hydroxydihydrodaidzein</td>
<td><img src="image" alt="Daidzein" /></td>
<td>272 mwt</td>
<td>possible precursor of demethylmedicarpin</td>
</tr>
</tbody>
</table>

* Toxicity was assessed by inhibition of spore germination using spores of *C. arachidicola* and several fungi not pathogenic to groundnut foliage.
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Seperation and identification of phytoalexins from leaves of groundnut (Arachis hypogaea) and development of a method for their determination by reversed-phase high-performance liquid chromatography

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ABSTRACT

Leaves of groundnut, Arachis hypogaea, infected with the early leaf spot fungus, Cercospora arachidicola, were extracted in aqueous ethanol and the phytoalexins partitioned into ethyl acetate. Flash chromatography of the ethyl acetate extract on silica gel yielded fractions with one to five compounds from which the phytoalexins could be isolated by semipreparative reversed-phase high-performance liquid chromatography (HPLC). The major phytoalexins were demethylmedicarpin, formononetin, 7,4’-dimethoxy-2’-hydroxyisoflavanone and medicarpin. Minor components were 7,2’-dihydroxy-4’-methoxyisoflavanone and daidzein. Compounds were identified by cochromatography and comparison of their ultraviolet and mass spectra with authentic samples using an HPLC system equipped with a diode-array detector, HPLC mass spectrometry and gas chromatography-mass spectrometry of their trimethylsilyl derivatives. A solid-phase extraction method was developed for processing large numbers of samples. Acetonitrile eluates from C18 cartridges were separated by reversed-phase HPLC and the phytoalexins quantified by reference to external standards of the authentic compounds.

INTRODUCTION

Phytoalexins are antimicrobial compounds which accumulate in plants in response to challenge by parasites and other traumas. In some interactions of plants and microbes there is good evidence that they are determinants of resistance [1,2]. We previously reported that the pterocarpan, medicarpin, was the predominant phytoalexin present in groundnut leaves infected by the fungus, Cercospora arachidicola [3]. However, further experiments showed that other antifungal compounds were also present. Before the role of medicarpin and these other phytoalexins in resistance to fungal leaf spot diseases could be assessed, it was necessary to identify them and devise means for their accurate determination.

In this paper, we report methods for phytoalexin isolation in quantities suffi-
cient for structural determination and a technique for their quantitative analysis in small samples involving solid-phase extraction and reversed-phase high-performance liquid chromatography (HPLC). Since low concentrations of phytoalexins were elicited by abiotic agents such as UV light or the salts of heavy metals, leaves infected with *C. arachidicola* were used as a source of the antifungal compounds.

**EXPERIMENTAL**

**Chemicals**

HPLC-grade acetonitrile and methanol were obtained from Fisons (Loughborough, UK). Ethyl acetate, methanol and cyclohexane (GPR) were purchased from BDH (Poole, UK). Chloroform and acetic acid (AR) were bought from May and Baker (Ongar, UK). Pure water was obtained from an Elga (High Wycombe, UK) pure-water system.

All other chemicals were of analytical grade and were purchased from BDH or Sigma (Poole, UK). Spherisorb ODS 1 (10 μm) was obtained from Phase Separations (Deeside, UK). Silica gel 60 and thin-layer chromatography (TLC) plates were obtained from BDH.

The phytoalexin standards demethylmedicarpin, medicarpin and 7,2'-dihydroxy-4'-methoxyisoflavanone were gifts from Professor P. M. Dewick (Department of Pharmacology, University of Nottingham, Nottingham, UK). Medicarpin, formononetin and daidzein were gifts from Professor W. Barz (Lehrstuhl für Biochemie der Pflanzen, Universität Münster, Münster, Germany).

**Extraction of phytoalexins**

**Method 1.** Groundnut leaves from the cultivars Flamingo and Egret (1 or 125 g fresh weight), infected with *C. arachidicola*, were collected from experimental plots near Harare, Zimbabwe and air-dried. They were vacuum-infiltrated with 60% ethanol and agitated at intervals for 24 h. After removal of the ethanol *in vacuo* at 40°C, the remaining aqueous solution was partitioned three times against ethyl acetate. The ethyl acetate fractions were combined and dried over anhydrous sodium sulphate.

**Method 2.** For analytical samples, leaves infected with *C. arachidicola* (1 g fresh weight) were extracted by facilitated diffusion[4] in 60% ethanol. The ethanol was decanted and diluted to 25% before being applied to a solid-phase cartridge. This consisted of 500 mg Techoprep C18 (25–40 μm: HPLC Technology, Macclesfield, UK), conditioned with methanol and washed with water. After application of the sample, the cartridge was washed with 5 ml of 25% ethanol, and the phytoalexins were eluted with 1 ml of acetonitrile.

The efficiency of these methods was examined by applying them to samples treated with silver nitrate, a poor elicitor of phytoalexins in groundnut leaves. Ten 5-μl droplets of silver nitrate solution (10^{-3}M) were applied to fully expanded, detached leaves and incubated for 48 h. Samples (1 g each), spiked with medicarpin (100 μg per sample) or not, were extracted by method 1 or method 2. In method 2, the cartridges were additionally eluted with a second millilitre of acetonitrile which was analysed separately. Material not retained on the cartridges was concentrated *in vacuo* and also analysed for the presence of medicarpin.
Isolation and purification of phytoalexins

Phytoalexins extracted by method 1 from large samples (125 g) were separated by a two-stage process consisting of flash chromatography [5] and semipreparative HPLC.

The ethyl acetate solution was evaporated to dryness in vacuo, the residue dissolved in 10 ml of chloroform and the chloroform solution separated on a column of silica gel 60 (Merck, 40–60 μm, 230–400 mesh, obtained from BDH; 150 mm × 20 mm). After conditioning the column with cyclohexane, the chloroform sample (5 ml) was applied and the phytoalexins eluted with a stepwise gradient of ethyl acetate in cyclohexane starting with 100% cyclohexane. At each step of the gradient (100 ml) the ethyl acetate content was increased by 10% (v/v), and two 50-ml fractions were cut. After the ethyl acetate concentration of the eluent had reached 50%, a final fraction consisting of 100 ml of 100% ethyl acetate was collected. All fractions were examined by analytical HPLC or TLC.

Phytoalexins in fractions from the silica column were isolated by semipreparative HPLC. The apparatus consisted of an Altex pump (Beckman Instruments, Berkeley, CA, USA), a column of Spherisorb ODS 1 (Phase Separations, 250 mm × 10 mm I.D.; 10 μm particle size), a Pye-Unicam UV detector (Philips Analytical, Cambridge, UK) set at 290 nm and a Tekman chart recorder (Tekman Electronics, Bicester, UK). Samples (250–500 μl) were introduced to the column via a 2-ml loop attached to an Altex valve and eluted in acetonitrile–water (1:1, v/v). Fractions corresponding to absorption peaks were collected manually. Purity of the compounds was checked by TLC using iodine vapour to visualize the spots and by HPLC using a system equipped with a diode-array detector (see below).

Thin-layer chromatography

Samples were run on TLC plates (silica gel 60 on an aluminium support; Merck No. 3554) in a tank saturated with a solvent system consisting of ethyl acetate–cyclohexane (1:1, v/v). Spots were detected by exposure to iodine vapour or spraying with diazotized p-nitroaniline [16], and those that were antifungal by spraying with a spore suspension of Cladosporium cucumerinum made up in half strength Czapek-Dox medium at a density of 0.8 absorbance units at 620 nm. After spraying, the plates were incubated at high humidity in the dark for 48 h [7].

Analytical HPLC

A Philips apparatus was used, consisting of two pumps (PU 4100), an automatic sampler (PU 4700) and a diode-array detector (PU 4021) interfaced to a trivector data system (Philips Analytical). Compounds were separated on a column of Spherisorb ODS 1 (250 mm × 4.6 mm I.D.; 10 μm particle size) protected by an Upchurch low-volume guard column (20 mm × 2 mm I.D., Anachem, Luton, UK) packed with Techoprep C18 (25–40 μm; HPLC Technology). The mobile phase was a gradient of acetonitrile in 1% acetic acid in which the acetonitrile concentration was increased from 35% to 40% over the first 12 min and then to 75% over the next 23 min. A reequilibration time of 5 min was allowed to elapse before the next sample was injected. The flow-rate was 1.5 ml/min and the eluent was monitored at 290 nm at an absorbance range of 0.04 a.u.f.s. Compounds were identified on the basis of retention time and comparison of their UV spectra with those of authentic samples. They were
Identification of phytoalexins

Ultraviolet spectroscopy. The UV spectra of purified compounds were obtained on a Varian-Cary spectrophotometer (Varian Assoc., Palo Alto, Ca, USA). Methanolic solutions were scanned from 220 to 340 nm.

Purified compounds were quantified by their absorbance using previously calculated molar extinction coefficients: medicarpin (287 nm), \( \log e = 3.90 \) [8]; isoflavones (277 nm), \( \log e = 4.00 \); formononetin (250 nm), \( \log e = 4.35 \) and demethylmedicarpin (287 nm), \( \log e = 3.93 \) [9].

Gas Chromatography–mass spectrometry (GC–MS). A Carlo Erba Strumentazione HPGC 5160 (Fisons Instruments, Crawley, UK) was fitted with a BP1 fused-silica/quartz capillary column (50 m x 0.32 mm I.D. with 0.5 \( \mu \)m film: SGE). The carrier gas, helium, was introduced into the system with a column head pressure of 130 KPa. Dried samples were derivatised in a mixture of trimethylchlorosilane (Sigma) and pyridine (1:1) and introduced into the instrument with a splitless on-column injector. The components were separated using the following GC programme: 50°C increasing to 250°C at 40°C/min with a further increase to 300°C at 5°C/min where it was maintained for 15 min. Ions were detected by a Hewlett-Packard 5970 series mass-selective detector (Hewlett Packard, Wokingham, UK) using an electron energy of 70 eV.

LC–MS. Samples were separated by gradient elution with a flow-rate of 1 ml/min on a Hichrom ODS 2 column (3 \( \mu \)m particle size; 150 mm x 4.9 mm I.D.). Solvent A was 20% acetonitrile in 0.1 \( M \) ammonium acetate and solvent B was 80% acetonitrile in 0.1 \( M \) ammonium acetate. Solvent B was maintained at 15% for 1 min, increased to 25% over the next 12 min and to 90% over the following 18 min. The column was reequilibrated by decreasing solvent B to 15% over 4 min and maintaining it at this percentage for the next 3 min. Analytes were monitored at 254 nm, and the mass spectra of the compounds giving rise to the peaks were determined by means of a Vestec 201 thermospray mass spectrometer with positive-ion discharge (1000 V: Vestec, Houston, TX, USA). Start conditions were: control, 144°C; tip heater, 288°C; source block, 326°C and vapour, 343°C.

HPLC–UV spectrophotometry. Samples were run on the analytical HPLC system and the retention times and spectra obtained from the diode-array detector compared with those of authentic compounds.

RESULTS

At least six antifungal zones that corresponded with areas of UV absorption were visible on TLC plates which had been spotted with extracts from diseased groundnut leaves and sprayed with spore suspension of \( C. cucumerinum \). Flash chromatography of extracts resulted in fractions containing one to five major components which could be separated by semipreparative HPLC (Fig. 1).

Medicarpin had an \( R_F \) of 0.71 on TLC and gave a pale orange spot when
Fig. 1. Distribution of phytoalexins in fractions from flash chromatography on silica gel. Two 50-ml fractions were collected for each concentration of ethyl acetate in the mobile phase apart from the final fraction which was 100 ml and was eluted with 100% ethyl acetate. 'Others' were unidentified compounds. Isoflavanone 3 was a compound with a UV spectrum and mass fragmentation pattern corresponding to an isoflavanone but was not characterized further. Isoflavanone 2 was identified as 7,2'-dihydroxy-4'-methoxyisoflavanone by GC–MS of the TMSi derivative, cochromatography by HPLC and matching of the UV spectrum with that of an authentic sample of the compound. Isoflavanone 1 was identified as 7,4'-dimethoxy-2'-hydroxyisoflavanone by GC–MS and LC–MS.

sprayed with diazotized p-nitroaniline. The absorption maxima in methanol were at 282 nm and 286.5 nm. GC–MS of the trimethylsilyl (TMSi) derivative gave m/z — 342 (M+1) and prominent peaks at 327 (M+1 — 15), 219 (M+1 — 123), 206 (M+1 — 136), 164 (M+1 — 178), 148 (M+1 — 194) and 73 (TMSi). On LC–MS the compound had a retention time of 24.72 min and gave an MH+ of 271. The retention time on the analytical HPLC system was 21.12 min compared with 21.10 min for an authentic sample, and good agreement of UV spectra was obtained.

Formononetin had an Rf of 0.42 on TLC and gave a very pale orange–yellow derivative when sprayed with diazotized p-nitroaniline. The absorption maxima in methanol were at 249 and 299 nm. GC–MS of the TMSi derivative gave m/z — 340 (M+1) with prominent peaks at 325 (M+1 — 15), 208 (M+1 — 132), 163 (M+1 — 177), 132 (M+1 — 208) and 73 (TMSi). An MH+ of 269 was obtained at a retention time of 21.72 min on LC–MS. On analytical HPLC an authentic sample eluted at 19.02 min and a compound with a similar spectrum from infected leaf samples at 19.10 min.

Demethylmedicarpin had an Rf value of 0.54 on TLC and gave a bright orange derivative on reaction with diazotized p-nitroaniline. The absorption spectra in methanol was similar to that of medicarpin with λmax at 282 and 286.5 nm. GC–MS of the TMSi derivative gave m/z 400 (M+1) with prominent peaks at 385 (M+1 — 15), 219 (M+1 — 181), 206 (M+1 — 194), 185 (M+1 — 215) and 73 (TMSi). LC–MS gave a peak at retention time 12.60 min with MH+ of 257. The authentic compound eluted at 11.20 min and a compound with a similar spectrum obtained from infected leaves at 11.10 min on the analytical HPLC.

An isoflavanone, referred to as isoflavanone 1, was identified as 7,4'-dimethoxy-2'-hydroxyisoflavanone, had an Rf value of 0.46 on TLC and produced a yellow–orange derivative when sprayed with diazotised p-nitroaniline. The absorption maxi-
ma in methanol were at 277 and 311 nm. GC-MS of the TMS derivative gave \( m/z \) 372 (\( M^+ \)) with prominent peaks at 357 (\( M^+ - 15 \)), 209 (\( M^+ - 163 \)), 193 (\( M^+ - 179 \)), 164 (\( M^+ - 208 \)), 149 (\( M^+ - 223 \)) and 121 (\( M^+ - 251 \)). LC-MS gave a peak at retention time 23.34 min with an \( MH^+ \) of 301. No authentic material was available for chromatographic and spectral comparison.

A second isoflavanone, referred to as isoflavanone 2, was identified as 7,2'-dihydroxy-4'-methoxyisoflavanone by co-chromatography and matching of the UV spectrum with that of authentic material. GC-MS of the TMS derivative gave \( m/z \) 430 (\( M^+ \)) with prominent peaks at 415 (\( M^+ - 15 \)), 281 (\( M^+ - 149 \)), 222 (\( M^+ - 208 \)) and 207 (\( M^+ - 223 \)). LC-MS gave a peak at a retention time of 15.14 min with \( MH^+ \) 287. The retention times of the authentic compound and samples from infected leaves on analytical HPLC were 13.38 and 13.42 min, respectively, and their spectra corresponded with absorption maxima at 275 and 311 nm.

An isoflavone was identified as daidzein. GC-MS of the TMS derivative gave \( m/z \) 398 (\( M^+ \)) with prominent peaks at 383 (\( M^+ - 15 \)), 281 (\( M^+ - 117 \)) and 207 (\( M^+ - 191 \)). LC-MS gave a peak at a retention time of 9.39 min with an \( MH^+ \) of 255. The authentic compound eluted at 9.10 min on the analytical HPLC system and a compound with a similar spectrum (\( \lambda_{max} \) 245 and 2989 nm) obtained from infected leaves eluted at 9.13 min.

Structures of the six compounds are presented in Fig. 2.

Analytical reversed-phase HPLC with a gradient of acetonitrile in 1% acetic acid on a column of Spherisorb ODS 1 (10 \( \mu \)m; 250 mm \( \times \) 4.6 mm I.D.) gave good separation of the principal phytoalexins (Fig. 3). Demethylmedicarpin, formonone-

Fig. 2. Structures of the phytoalexins identified in leaves of groundnuts infected by the fungal parasite \( \textit{C. arachidicola} \).
Fig. 3. HPLC of phytoalexins found in leaves of groundnuts infected by the fungal parasite C. arachidicola. (A) Chromatogram of six phytoalexin standards; a 10-μl sample containing the following concentrations of phytoalexins was injected: 1, daidzein, 50 μg/ml; 2, demethylmedicarpin, 25 μg/ml; 3, isoflavanone 2 (7,2'-dihydroxy-4'-methoxyisoflavanone), 10 μg/ml; 4, formononetin, 150 μg/ml; 5, isoflavanone 1 (7,4'di-methoxy-2'-hydroxyisoflavanone), 65 μg/ml; 6, medicarpin 100 μg/ml. (B) Typical chromatogram of an extract from infected groundnut leaves with peaks labelled as in (A). (C) Typical chromatogram of an extract from uninfected groundnut leaves showing traces of medicarpin only.
TABLE I

CONCENTRATIONS OF PHYTOALEXINS ACCUMULATED IN UNINFECTED AND INFECTED GROUNDNUT LEAVES (CV. EGRET) 18 DAYS AFTER INOCULATION WITH CERCOSPO-RA ARACHIDICOLA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mean ± S.D., n=5) (µg/g fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
</tr>
<tr>
<td>Daidzein</td>
<td>8.45 ± 3.98</td>
</tr>
<tr>
<td>Demethylmedicarpin</td>
<td>57.50 ± 26.25</td>
</tr>
<tr>
<td>Isoflavanone 2*</td>
<td>3.70 ± 1.84</td>
</tr>
<tr>
<td>Formononetin</td>
<td>127.29 ± 7.79</td>
</tr>
<tr>
<td>Isoflavanone 1*</td>
<td>53.82 ± 14.62</td>
</tr>
<tr>
<td>Medicarpin</td>
<td>122.10 ± 25.75</td>
</tr>
</tbody>
</table>

* Isoflavanone 2 was identified as 7,2'-dihydroxy-4'-methoxyisoflavanone by GC-MS of the TMSi derivative, co-chromatography by HPLC and matching of the UV spectrum with that of an authentic sample of the compound.

* Isoflavanone 1 was identified as 7,4'-dimethoxy-2'-hydroxyisoflavanone by GC-MS and LC-MS.

tin, 7,4'-dimethoxy-2'-hydroxyisoflavanone and medicarpin were prominent compounds in extracts of groundnut leaves (cv. Egret) infected with C. arachidicola and harvested 18 days later. Daidzein and 7,2'-dihydroxy-4'-methoxyisoflavanone were minor components. The phytoalexins were essentially absent from uninfected leaves apart from low concentrations of medicarpin (Fig. 3 and Table I).

Method 2, using solid-phase extraction, was as efficient as method 1 for the extraction of phytoalexins and more appropriate for the analysis of large numbers of samples (Table II). In tests of samples spiked with medicarpin, most of the compound

TABLE II

COMPARISON OF RECOVERY OF MEDICARPIN FROM LEAF EXTRACTS BY LIQUID-LIQUID AND SOLID-PHASE EXTRACTION

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Medicarpin (mean ± S.D., n=3) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid-liquid extraction</td>
<td></td>
</tr>
<tr>
<td>Unspiked sample</td>
<td>12.55 ± 4.79</td>
</tr>
<tr>
<td>Spiked sample</td>
<td>114.71 ± 4.68</td>
</tr>
<tr>
<td>Solid-phase extraction</td>
<td></td>
</tr>
<tr>
<td>Unretained eluate</td>
<td>0.00</td>
</tr>
<tr>
<td>Unretained eluate (spiked)</td>
<td>0.00</td>
</tr>
<tr>
<td>First ml of acetonitrile eluate (unspiked)</td>
<td>22.74 ± 5.57</td>
</tr>
<tr>
<td>First ml of acetonitrile eluate (spiked)</td>
<td>120.98 ± 5.65</td>
</tr>
<tr>
<td>Second ml of acetonitrile eluate (unspiked)</td>
<td>0.00</td>
</tr>
<tr>
<td>Second ml of acetonitrile eluate (spiked)</td>
<td>3.99 ± 3.92</td>
</tr>
</tbody>
</table>

* Unspiked samples were extracts of leaves which had been treated with silver nitrate (see text for details).

* Spiked samples were extracts of leaves which had been treated with silver nitrate and spiked with 100 µg of medicarpin (see text for details).
was eluted in the first millilitre of acetonitrile from the solid-phase cartridge and <4% in the second millilitre. In view of this result and the good recovery in the first millilitre of eluate a single millilitre would seem appropriate for routine work.

DISCUSSION

Accumulation of isoflavone and isoflavanone phytoalexins is a common response of legumes to challenge with phytopathogenic fungi [10,11]. The identification of the compounds reported in this paper (Fig. 2) was therefore not surprising although they contrast with the stilbenes elicited in groundnut cotyledons [12,13]. Formononetin, daidzein and 7,2'-dihydroxy-4'-methoxyisoflavanone are precursors of medicarpin [14,15], 4'-methylation of daidzein results in the formation of formononetin which is 2'-hydroxylated and reduced to the isoflavanone. Cyclisation of this compound leads to the formation of medicarpin. In contrast, demethylmedicarpin by analogy with the demethylation of pisatin [2] and comparison with the modification of medicarpin by the fungus, _Ascochyta rabiei_ [16], is probably a degradation product.

With regard to the techniques described in this paper, the use of flash chromatography was a helpful first stage in the purification of phytoalexins and resulted in fractions containing one to five compounds. These were easily separated on semipreparative HPLC with an isocratic mobile phase. For the routine analysis of phytoalexins in small samples from infected groundnut leaves, solid-phase extraction proved to be a practical and reliable alternative to liquid–liquid extraction. Although reasonable separation was achieved on Sherisorb ODS 1 (10 μm) there is no doubt that the use of a smaller size of particle in the stationary phase would enhance resolution.

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