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Biotic and abiotic elicitation of phytoalexins in leaves of groundnut (*Arachis hypogaea* L.)*

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Groundnut leaves (cultivar TMV2) infected with the fungal pathogens, *Cercospora arachidicola*, *Phaeoisariopsis personata* and *Puccinia arachidis* accumulated 1830, 664 and 162 nmol phytoalexins g^{-1} fresh wt., respectively, 4 weeks after inoculation, whereas leaves infested with *Frankliniella* sp. for the same period contained 1·25 nmol phytoalexins g^{-1} fresh wt. Spraying abraded leaves with salicylic acid (0·01 M) resulted in the accumulation of 1270 nmol phytoalexins g^{-1} fresh wt. 120 h after treatment and irradiation of abaxial leaf surfaces with u.v. light (254 nm) for 48 h and incubation in the dark for a further 96 h caused the accumulation of 393 nmol phytoalexins g^{-1} fresh wt. Compounds with u.v. spectra corresponding to isoflavanones were almost exclusively synthesized in response to abiotic elicitors but in leaves infected with fungal pathogens formononetin, daidzein, and medicarpin were also present, though as minor components.

INTRODUCTION

Infection of groundnut (*Arachis hypogaea* L.) leaves by *Cercospora arachidicola* Hori and *Phaeoisariopsis personata* (Berk. & Curt.) v. Arx, causal agents of early and late leaf spot, respectively, may result in yield losses of over 50% [*14*]. Additionally, when these diseases are associated with rust, caused by *Puccinia arachidis* Speg., losses may rise to 70% [*22*]. Insect pests such as thrips (*Frankliniella* sp.) may damage large areas of the leaf, e.g. 47% [*15*], and also act as vectors of tomato spotted wilt virus [*18*]. Chemical control, although effective, is expensive, potentially harmful to the environment and impracticable for poor farmers in developing countries. A further disadvantage of pesticides is that their frequent application may lead to the development of tolerance in the target organism [*20*]. An alternative approach is to develop resistant cultivars. To date, many thousands of groundnut genotypes have been screened and the more resistant ones included in breeding programmes which have resulted in the release of cultivars with high levels of resistance [*26*].

An approach, which is complementary to screening and breeding, is to select genotypes that have efficient mechanisms of defence. One such mechanism is the

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Abbreviations used in text: Diam, diameter; EC, end-capped; HPLC, high performance liquid chromatography; RT, retention time; sh, shoulder.

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phytoalexin response. Over 20 compounds have been identified as phytoalexins in groundnuts but their elicitation has received little attention [*21*]. Phytoalexins may be elicited by biotic agents such as plant pathogenic fungi but their presence complicates the results since not only may they elicit phytoalexins but also degrade them. This problem can be circumvented by the use of physical or chemical agents as abiotic elicitors. Physical elicitors include local freezing and thawing [*8*, *16*] and u.v. irradiation [*13*]. Chemical elicitors may be derived from pathogens e.g. the hepta-β-glucoside from *Phytophthora megasperma* [*2*] or may be of abiotic origin e.g. the salts of heavy metals. Salicylic acid induces both local and systemic resistance in plants but this resistance is mediated, at least in part, through the production of pathogenesis-related (PR)-proteins some of which are chitinases or glucanases [*12*, *17*]. Few reports have suggested a role for salicylic acid in phytoalexin elicitation. One exception concerns the enhancement of phytoalexin secretion in cell suspension cultures of parsley suboptimally elicited with a cell wall preparation from *Phytophthora megasperma* f. sp. *glycinea* [*10*].

As a preliminary to testing the ability of a number of cultivars of groundnut for their potential to synthesize and accumulate a range of phytoalexins, a single cultivar, TMV2, which is widely grown in southern India, was challenged by four biotic agents (*C. arachidicola*, *P. personata*, *P. arachidis* and *Frankliniella* sp.) and two abiotic agents (short wave u.v. and salicylic acid) and the phytoalexins accumulated determined by HPLC. The abiotic elicitors were included in order to compare their elicitor activity with that of the pathogens as well as to avoid the possibility of phytoalexin metabolism by organisms other than the plant.

MATERIALS AND METHODS

Plant material

Groundnuts (cv. TMV2) were sown in John Innes No. 3 compost in 10 cm pots (one seed per pot). The pots were placed in a greenhouse maintained at 23 ± 2 °C and were watered every second day. Plants were sprayed with an insecticide (Sybol, ICI, U.K.) as required, to protect them from thrips.

Fungal material

Spores of *Cercospora arachidicola*, *Phaeoisariopsis personata* and *Puccinia arachidis* harvested from naturally infected groundnut plants in experimental trials at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India, were multiplied on leaves of the disease-susceptible cv. TMV2. Spores were harvested using a cyclone spore collector (ERI Instrument Shop, Iowa State University, Ames, U.S.A.) and dry spores were stored in glass tubes at 4 °C in a refrigerator until required for use [*24*].

Inoculation and scoring of symptoms

Spore suspensions of *C. arachidicola* (10^4 and 10^3 spores ml^{-1}), *P. personata* and *P. arachidis* (both 10^4 ml^{-1}) in 1 % Tween-80 (polyoxyethylenesorbitan monooleate) were sprayed onto leaves of 3-week-old plants to run-off. Plants inoculated with *P. arachidis* were enclosed for 24 h in plastic bags which had been wetted by spraying their inner surfaces with distilled water. For *C. arachidicola* and *P. personata*, plants were sprayed with

distilled water in the evenings and covered with plastic bags for five consecutive nights of 16 h and allowed to dry during the day thus providing a total intermittent leaf wetness for 80 h [*24*]. Four weeks after inoculation, lesion numbers cm^{-2} of the lamina and their diameters (25 per replication) were recorded.

Insect damage

Leaves of 3 week-old plants were naturally infested by tobacco thrips (*Frankliniella* sp.).

Ultraviolet light treatment

Leaves were detached from 3-week-old plants at their pulvinus and their petioles covered with moist tissue paper. They were arranged in plastic trays (24·5 × 31·5 cm) abaxial or adaxial surface uppermost and left for 48 h on the base of a Chromato-Vue Cabinet (Model CC-20, Ultra Violet Products Inc., San Gabriel, U.S.A.) 20 cm below the short wave u.v. lamp (254 nm: 180 $\mu W\ cm^{-2}$: CP Instrument Co., U.K.). Control leaves were removed and treated in the same way except that they were kept in the dark. After the 48 h period, all leaves were incubated in the dark at room temperature (25 ± 2 °C) for 96 h at high humidity.

Salicylic acid treatment

The adaxial surface of leaves from 3-week-old plants was injured by rubbing carborundum powder gently on the leaf surface with a finger (in a surgical glove) and the leaves washed with distilled water. After blotting with tissue paper, salicylic acid (0·01 M) was applied twice at two-day intervals as a spray. Alternatively, salicylic acid (0·01 M) was applied as a root drench (20 ml per pot) twice at two-day intervals. A set of untreated control plants and another set of plants treated with carborundum and sprayed with water instead of salicylic acid were included in the experiment. All treatments were replicated three times.

Extraction of phytoalexins

Leaf samples were homogenized in 60% ethanol (1 g fresh wt. 15 ml^{-1}) and placed in the dark at room temperature for 1 h. Samples were filtered through GFA filter cups (Whatman, U.K.) and the alcohol was removed by rotary evaporation at 35 °C. After centrifuging at 30000 ***g*** for 15 min, phytoalexins were extracted from the supernatant using C18 (EC) Isolute cartridges (1 g; Jones Chromatography, U.K.). The cartridges were solvated in 6 ml ethanol followed by 6 ml H_2O and samples were applied in 10% ethanol. After washing with 10% ethanol (6 ml) the phytoalexins were eluted in two fractions (3 ml each) of 60% acetonitrile. The acetonitrile eluates were combined and diluted to 10% acetonitrile with distilled water before loading onto another C18 cartridge, solvated as described earlier. Phytoalexins were eluted in two fractions (1 ml each) of 100% acetonitrile, combined and stored in clear glass sample vials (7 ml: Merck, U.K.).

Bioassay

Eluants from the second C18 cartridge, equivalent to 450 mg fresh weight of leaves, were tested for antifungal compounds by a TLC-bioassay using *Cladosporium cladosporioides* (IMI 045534) as indicator organism [*9*].

HPLC analysis

Samples were analysed by HPLC (Unicam, U.K.) equipped with a PU4100 quaternary pump, a Rheodyne 7125 injection valve with a 20 μl loop and PU4021 diode array detector connected to a 486 personal computer (Olympic Technology, U.K.) containing PU6003 diode array and PU6000 integration software. Chromatography was performed on a 250 × 4·6 mm reverse phase Finesse column (Jones Chromatography, U.K.) with Spherisorb ODS2 (5 μm) packing. A guard column (20 × 4·6 mm) of identical packing was used to protect the analytical column. The column temperature was maintained at 46 °C using a block heater (Jones Chromatography, U.K.). Phytoalexins were eluted by a gradient of acetonitrile in 5 % formic acid at a flow rate of 0·8 ml min^{-1} in which the acetonitrile concentration was increased from 30 to 100 % in six steps: 1, 30–35 % in 5 min; 2, 35 % for 5 min; 3, 35–49 % in 15 min; 4, 49 % for 5 min; 5, 49–60 % in 10 min and 6, 60–100 % in 5 min. A re-equilibration time of 30 min was allowed to elapse before the next sample was injected. Compounds were identified on the bases of their retention times and matching of their u.v. spectra with those of authentic standards. Identified compounds were quantified using the PU6000 integration system software by comparison of peak areas with those of external standards. Phytoalexin standards of formononetin and daidzein were obtained from Apin Chemicals Ltd. (Oxford, U.K.). Medicarpin and 7,4′-dimethoxy-2′hydroxy-isoflavanone (referred to as isoflavanone 3) were isolated and purified in the laboratory. Standard solutions were obtained gravimetrically (daidzein and formononetin) or by reference to their extinction coefficients (medicarpin and isoflavanone 3). Compounds with u.v. spectra corresponding to isoflavanone 3 (compounds referred to as isoflavanone 1, 2, 4 and 5) were quantified using the same extinction coefficient as this compound (277 nm) $\log \varepsilon = 4{\cdot}00$ [*5*]. Four concentrations of each phytoalexin ranging from 50 to 1500 μg ml^{-1} were run on the HPLC in order to determine the linearity of the detector response and calculate response factors. These were then used to calculate the phytoalexin concentrations in samples expressed as nmol g^{-1} fresh wt.

RESULTS

Symptoms

Leaves inoculated with 10^3 spores of *C. arachidicola* ml^{-1} had the fewest lesions (1·60 cm^{-2}) but they were the largest (3·74 mm diam: Table 1). Those inoculated with 10^4 spores ml^{-1} of this pathogen had over twice as many lesions but they were smaller (2·36 mm diam) and similar in both number and size to those caused by *P. personata* (Table 1). Rust lesions, caused by *P. arachidis*, were the most numerous (7·86 cm^{-2}) but they were also the smallest (0·88 mm diam; Table 1).

Leaves irradiated adaxially or abaxially with short wave u.v. light (254 nm) for 48 h and incubated in the dark for a further 96 h fluoresced blue when observed under long wave u.v. light (365 nm).

Leaves abraded with carborundum and sprayed with salicylic acid (0·01 M) became necrotic but abraded leaves sprayed with water showed little necrosis.

TABLE 1

Lesion numbers (cm^{-2} leaf area)[a], lesion sizes (mm diameter)[b] and total phytoalexin concentrations[a] of groundnut leaves (cv. TMV2) challenged by three fungal pathogens (Cercospora arachidicola *causing early leaf spot*, Phaeoisariopsis personata *causing late leaf spot and* Puccinia arachidis *causing rust*) *and two abiotic agents (u.v. light-254 nm and salicylic acid-0·01 M).*

Disease/ Treatment	Inoculum concentration (number of spores ml^{-1})	Lesion number cm^{-2} leaf area	Lesion size (mm in diameter)	Total phytoalexin (nmol g^{-1} fresh wt.)
Early leafspot (S)	10000	3·75 (±0·58)	2·36 (±0·93)	1830·21 (±511·52)
Early leafspot (M)	1000	1·60 (±0·59)	3·74 (±0·98)	745·17 (±48·01)
Late leafspot	10000	3·33 (±0·56)	2·84 (±1·12)	663·57 (±85·65)
Rust	10000	7·86 (±1·26)	0·88 (±0·29)	161·59 (±16·62)
Control	—	—	—	2·96 (±5·13)
u.v.-control	—	—	—	103·87 (±68·79)
u.v.-adaxial	—	—	—	115·11 (±44·67)
u.v.-abaxial	—	—	—	393·44 (±58·42)
Carbo-control	—	—	—	174·43 (±120·31)
SA-Root drench	—	—	—	590·48 (±25·08)
SA-Foliar spray	—	—	—	1270·47 (±137·69)

[a]mean of three replicates

[b]mean of three replicates and 25 lesions per replicate

(M), mild infection; (S), severe infection; SA, salicylic acid; —, not inoculated; figures in parentheses are standard deviations.

Bioassay of antifungal compounds from groundnut leaves

Antifungal compounds from leaf extracts were retained on C18 (EC) cartridges when loaded in 10% ethanol and were eluted in 60% acetonitrile. The compounds were also retained on C18 (EC) cartridges when the 60% acetonitrile fraction was diluted to 10% acetonitrile and were eluted in 100% acetonitrile (2 ml).

All four biotic treatments and both abiotic treatments elicited antifungal compounds in groundnut leaves (Fig. 1). Of the four biotic treatments, greatest antifungal activity was found in extracts from leaves infected with *C. arachidicola*, showing mild or severe symptoms, and least from leaves infested with *Frankliniella* sp. Extracts of leaves infected with either *Puccinia arachidis* or *Phaeoisariopsis personata* had intermediate activity (Fig. 1). Irradiation of abaxial leaf surfaces with shortwave u.v. elicited greater antifungal activity than leaves irradiated adaxially. Application of salicylic acid as a foliar spray was more effective than application as a root drench. Little or no antifungal activity was found in any of the three controls, uninoculated leaves, leaves incubated without exposure to u.v. light and abraded leaves treated with water (Fig. 1).

Identification of phytoalexins

Eight compounds that were either known phytoalexins or appeared by their spectra to be closely related to known phytoalexins were found in extracts from leaves treated with biotic and abiotic elicitors (Table 2). Of these, four were identified on the bases of matching retention times and u.v. spectra. They were daidzein (peak 1), formononetin (peak 4), isoflavanone 3 (7,4′-dimethoxy-2′hydroxyisoflavanone) (peak 5), and medicarpin (peak 6) (Fig. 2). The u.v. spectra of peaks 2, 3 and 8 as well as 7 (not shown in Fig. 2 as it is found only in abraded leaves and those treated with

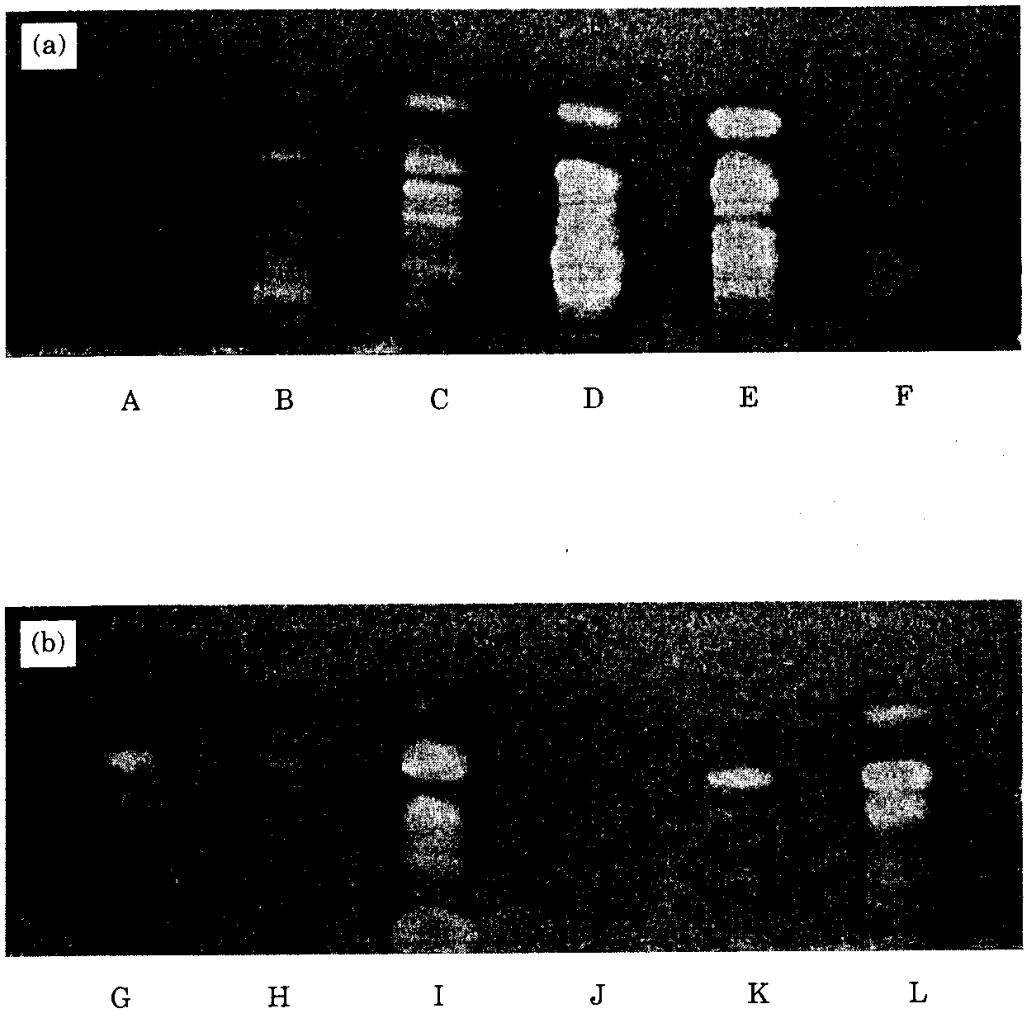

FIG. 1. Thin layer chromatography bioassay demonstrating antifungal activity of extracts from groundnut leaves (cv. TMV2) challenged by (a) four biotic agents and (b) two abiotic agents. (a) Left to right: A, healthy leaves; B, *Puccinia arachidis*; C, *Phaeoisariopsis personata*; D, *Cercospora arachidicola* mild infection; E, *C. arachidicola* severe infection; F, *Frankliniella* sp. (b) G, u.v. exposure of adaxial leaf surface; H, control treatment for the u.v. experiment; I, u.v. exposure of abaxial leaf surface; J, leaves sprayed with water only; K, salicylic acid applied as a root drench; L, salicylic acid applied as foliar spray. All samples represent 450 mg fresh wt. of leaf tissue. Samples were spotted on a TLC plate (precoated with silica gel F_{254}) and chromatographed in cyclohexane and ethyl acetate (1 : 1 v/v) before spraying with a spore suspension of *Cladosporium cladosporioides* in double strength Czapek Dox liquid medium and incubating at 25 °C for 48 h at high humidity. Zones of antifungal activity appear as white areas on a grey background.

salicylic acid) were very similar to that of isoflavanone 3, giving matches of 92·0–99·5 % when compared by the method of least squares. Pending identification by mass spectrometry and nuclear magnetic resonance spectroscopy of the isoflavanone-like compounds corresponding to peaks 2, 3, 7 and 8, these are referred to as isoflavanones 1, 2, 4 and 5, respectively. Other peaks, the most prominent of which had a retention time of 12·02 min and was found in leaves severely infected with *C. arachidicola* and *P. personata*, were not identified.

Comparison of biotic elicitors

The phytoalexins accumulated in leaves infected by the three fungal pathogens differed qualitatively and quantitatively. Extracts from leaves severely infected with *C. arachidicola* (10^4 spores ml^{-1}) contained the greatest total amount of phytoalexins

TABLE 2
Retention times and spectral characteristics of phytoalexins and related compounds elicited in leaves of groundnut (cv. TMV2).

Peak no.[a]	Compound name	Retention time (min)[b]	Spectral characteristics	
			λ max (nm)	Shoulder (nm)
1	Daidzein	8·04	254	299
2	Isoflavanone like compound (Isoflavanone 1)	9·28	280	309
3	Isoflavanone like compound (Isoflavanone 2)	9·92	280	309
4	Formononetin	17·89	253	303
5	7,4′-dimethoxy-2′hydroxyisoflavanone (Isoflavanone 3)	19·90	280	309
6	Medicarpin	21·70	287	—
7	Isoflavanone like compound (Isoflavanone 4)	25·92	280	309
8	Isoflavanone like compound (Isoflavanone 5)	26·13	280	309

[a]Refer to Fig. 2 for chromatogram
[b]See materials and methods for HPLC conditions
— no shoulder.

(1830 nmol g^{-1} fresh wt.), those infected with *P. arachidis* the least (162 nmol g^{-1} fresh wt.) and extracts from leaves infected with *P. personata* or mildly infected with *C. arachidicola* (10^3 spores ml^{-1}) were intermediate (664 and 745 nmol, respectively) (Tables 1 and 3 and Fig. 3). Control leaves and those infested by *Frankliniella* sp. accumulated negligible amounts of phytoalexin (Table 3). Isoflavanone 3 and isoflavanone-like compounds accounted for 57 % or more of the identified phytoalexins accumulated in response to biotic treatments (Fig. 3). The other compounds identified were daidzein, formononetin and medicarpin (Tables 2 and 3). Leaves infected by *P. arachidis* only accumulated isoflavanones 3 and 5, but those infected with *C. arachidicola* and *P. personata* accumulated isoflavanones 1 and 2 as well (Table 3).

Comparison of abiotic elicitors

The phytoalexins elicited abiotically also varied qualitatively and quantitatively. The most effective elicitor was salicylic acid (0·01 M) applied as a foliar spray to abraded leaves, giving a total phytoalexin concentration of 1270 nmol g^{-1} fresh wt. whereas, when applied as a root drench, 590 nmol phytoalexins g^{-1} fresh wt. were obtained. Irradiation of the abaxial leaf surface with u.v. gave 393 nmol phytoalexin g^{-1} fresh wt. whereas irradiation of the adaxial leaf surface gave values that were not significantly different from controls (115 nmol phytoalexin g^{-1} fresh wt. compared with 104 nmol g^{-1} fresh wt.; Table 4 and Fig. 3). Isoflavanones accounted for > 88 % of the identified phytoalexins elicited abiotically, only small amounts (< 12 %) of other phytoalexins, daidzein, formononetin and medicarpin being present (Table 4 and Fig. 3). Of the five isoflavanones, only isoflavanones 3, 4 and 5 were accumulated in leaves

TABLE 3

Phytoalexins (nmol g^{-1} fresh wt.[a]) elicited in groundnut leaves by four biotic agents (Cercospora arachidicola, Phaeoisariopsis personata, Puccinia arachidis *and* Frankliniella *sp.*).

Treatment	Iso 1[b]	Iso 2[b]	Iso 3[c]	Iso 4[b]	Iso 5[b]	Total isoflavanones	Others[d]	Total phytoalexins
C. arachidicola (S)	937·96 (±322·00)	213·22 (±126·56)	125·46 (±11·80)	0·00	96·45 (±16·22)	1373·09 (±423·77)	457·11 (±89·03)	1830·21 (±511·52)
C. arachidicola (M)	150·90 (±34·68)	51·00 (±13·04)	104·96 (±63·06)	0·00	116·26 (±47·11)	423·13 (±21·98)	322·04 (±26·96)	745·17 (±48·01)
P. personata	173·87 (±50·42)	39·58 (±8·87)	83·54 (±18·25)	0·00	82·39 (±17·16)	379·38 (±41·89)	284·19 (±44·00)	663·57 (±85·65)
P. arachidis	0·00	0·00	21·67 (±1·37)	0·00	76·87 (±12·18)	98·84 (±12·60)	62·75 (±9·55)	161·59 (±16·62)
Frankliniella sp.	0·00	0·00	1·25 (±2·17)	0·00	0·00	1·25 (±2·17)	0·00	1·25 (±2·17)
Control	0·00	0·00	1·92 (±3·33)	0·00	0·00	1·92 (±3·33)	1·04 (±1·81)	2·96 (±5·13)

[a]mean of three replicates

[b]Iso 1, 2, 4 and 5 are isoflavanone-like compounds corresponding to peaks 2, 3, 7 and 8 (Fig. 2)

[c]7,4′-dimethoxy-2′hydroxyisoflavanone

[d]daidzein, formononetin and medicarpin

S, severe infection (inoculated with 10^4 spores ml^{-1}); M, mild infection (inoculated with 10^3 spores ml^{-1}); figures in parentheses are standard deviations.

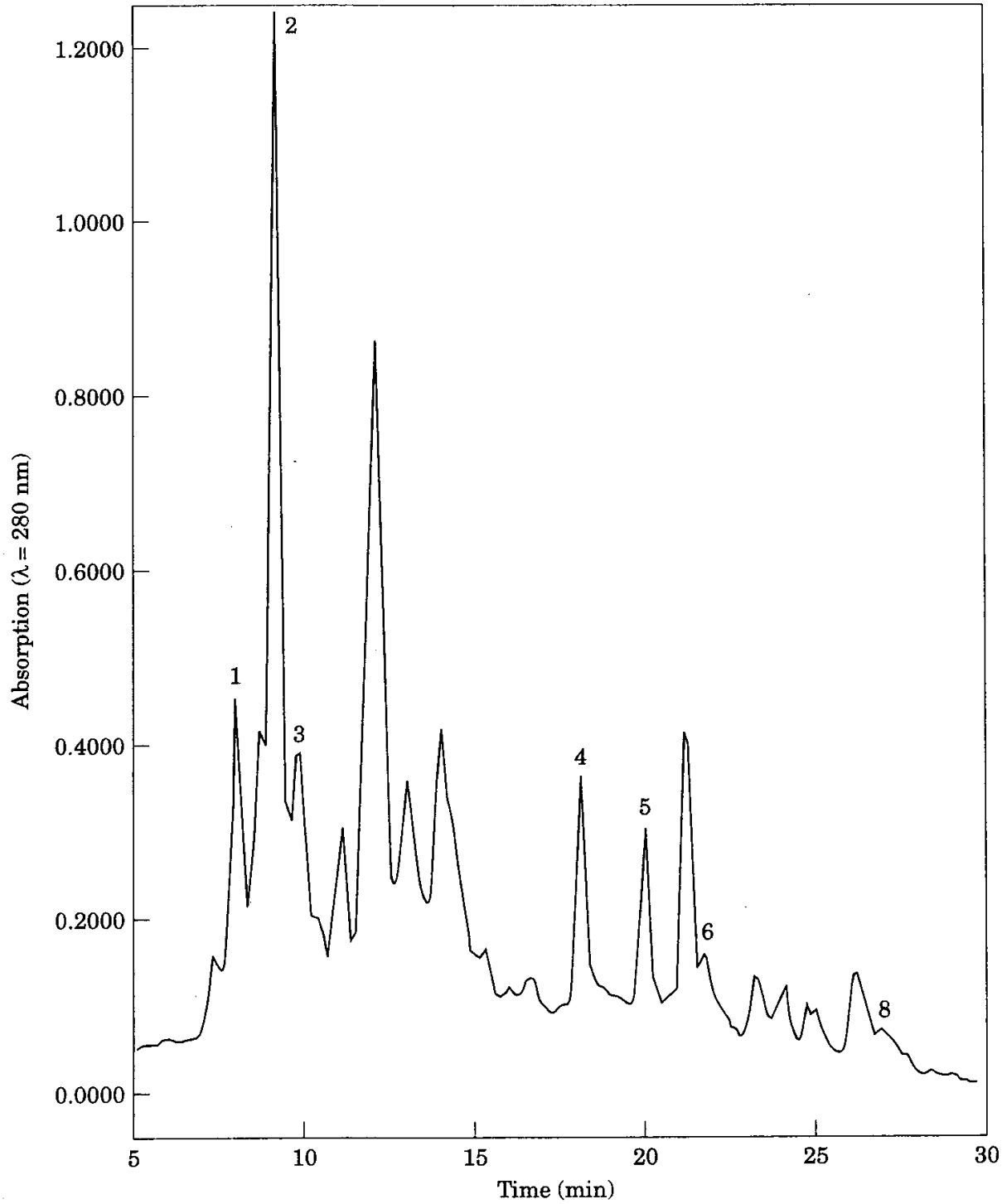

FIG. 2. Chromatogram of extract from leaves inoculated with *Cercospora arachidicola* (10^4 spores ml^{-1}). Time in minutes is represented on the x-axis and absorption (λ = 280 nm) on the y-axis. Peak 1, daidzein; peak 2, isoflavanone 1; peak 3, isoflavanone 2; peak 4, formononetin; peak 5, isoflavanone 3; peak 6, medicarpin; peak 8, isoflavanone 5. A further isoflavanone-like compound which eluted at 25·90 min (found in abraded leaves or those treated with salicylic acid but not in infected leaves) was designated isoflavanone 4. Unlabelled peaks were not identified.

treated abiotically. Irradiation with short wavelength u.v. light (254 nm) elicited only isoflavanone 3 whereas treatment with salicylic acid elicited isoflavanones 3, 4 and 5 (Table 4).

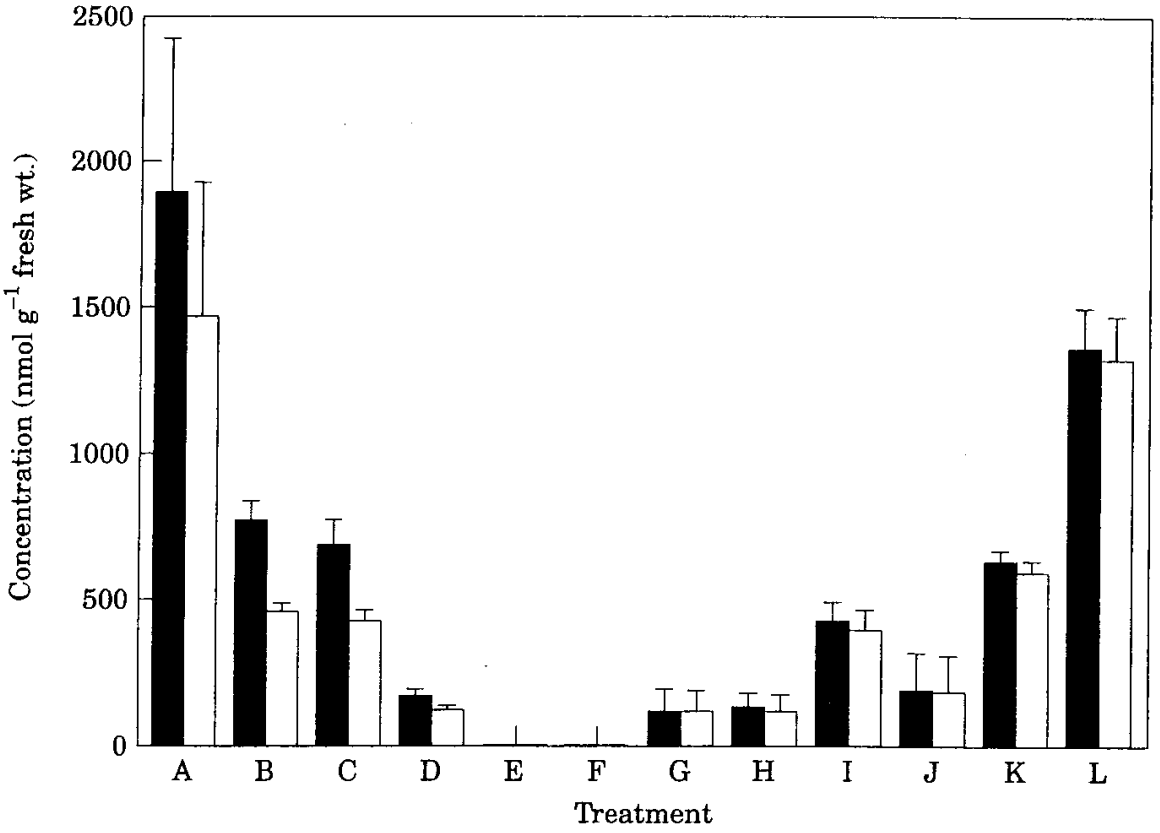

FIG. 3. Comparison of total concentrations of phytoalexins and the isoflavanone component (nmol g^{-1} fresh wt.) in extracts of 11 samples of groundnut leaves challenged by different agents. Data are means of three replicates. Vertical bars are standard deviations. A, *Cercospora arachidicola* (severe infection); B, *C. arachidicola* (mild infection); C, *Phaeoisariopsis personata*; D, *Puccinia arachidis*; E, *Frankliniella* sp.; F, healthy control; G, control treatment for the u.v. experiment; H, u.v. exposure (254 nm) of the adaxial leaf surfaces; I, u.v. exposure (254 nm) of the abaxial leaf surfaces; J, carborundum control; K, salicylic acid (0·01 M) (root drench); L, salicylic acid (0·01 M) (foliar spray). (■) total phytoalexins; (□) total isoflavanones.

Comparison of biotic with abiotic elicitation of phytoalexins

The total concentration of identified phytoalexins in abraded groundnut leaves sprayed with salicylic acid was > 69% of that elicited by a severe infection of *C. arachidicola* (1270 nmol g^{-1} fresh wt. compared with 1830 nmol g^{-1} fresh wt.; Table 1 and Fig. 4). However, the difference is mostly attributable to daidzein, formononetin and medicarpin, the overall isoflavanone content being similar although concentrations of individual isoflavanones differed (Tables 3, 4 and Fig. 4). Daidzein, formononetin and medicarpin accounted for 25–43% of the total phytoalexin concentration in the biotic treatments while they accounted for < 12% in the abiotic treatments (Tables 3 and 4). Within the isoflavanone component the main difference between biotic and abiotic treatments was in the polarity of the compounds elicited (Tables 3 and 4). Isoflavanone 1 was the major component in leaves infected with *C. arachidicola* and *P. personata*, which also had lesser amounts of isoflavanone 2, 3 and 5 but no isoflavanone 4. In contrast, isoflavanone 3 was the major component in the abiotic treatments and none of the abiotic treatments elicited isoflavanones 1 and 2. Salicylic acid applied as a spray or as a root drench elicited isoflavanones 4 and 5 but these were not present in u.v.-irradiated leaves. *P. arachidis* elicited low concentrations of isoflavanone

TABLE 4

Phytoalexins (nmol g^{-1} fresh wt.[a]) elicited in groundnut leaves by two abiotic agents (u.v. light-254 nm and salicylic acid-0·01 M).

Treatment	Iso 1[b]	Iso 2[b]	Iso 3[c]	Iso 4[b]	Iso 5[b]	Total isoflavanones	Others[d]	Total phytoalexins
u.v.-control	0·00	0·00	102·81 (±68·67)	0·00	0·00	102·81 (±68·67)	1·06 (±1·83)	103·87 (±68·79)
u.v.-adaxial	0·00	0·00	101·28 (±45·07)	0·00	0·00	101·28 (±45·07)	13·83 (±5·34)	115·11 (±44·67)
u.v.-abaxial	0·00	0·00	362·14 (±61·45)	0·00	0·00	362·14 (±61·45)	31·30 (±5·64)	393·44 (±58·42)
SA-control	0·00	0·00	1·92 (±3·33)	0·00	0·00	1·92 (±3·33)	1·20 (±1·68)	3·12 (±5·00)
Carbo-control	0·00	0·00	140·21 (±68·41)	28·40 (±49·20)	0·00	168·61 (±117·18)	5·82 (±3·14)	174·43 (±120·31)
SA-RTD	0·00	0·00	336·64 (±12·34)	65·21 (±12·32)	143·97 (±26·75)	545·84 (±25·80)	44·65 (±5·94)	590·48 (±25·08)
SA-FS	0·00	0·00	839·02 (±93·56)	128·00 (±26·47)	264·16 (±34·99)	1231·17 (±139·71)	39·30 (±2·17)	1270·47 (±137·69)

[a]mean of three replicates

[b]Iso 1, 2, 4 and 5 are isoflavanone-like compounds corresponding to peaks 2, 3, 7 and 8 (Fig. 2)

[c]7,4′-dimethoxy-2′hydroxyisoflavanone

[d]daidzein, formononetin and medicarpin

SA-control: leaves with water spray only; Carbo-control, leaves with carborundum injury only; SA, salicylic acid; RTD, root drench; FS, foliar spray; figures in parentheses are standard deviations.

phytoalexins, consisting of isoflavanones 3 and 5 (22 and 77 nmol g^{-1} fresh wt., respectively) (Tables 3 and 4). Controls for the u.v. experiments had higher concentrations of phytoalexins than those of controls for the biotic experiments.

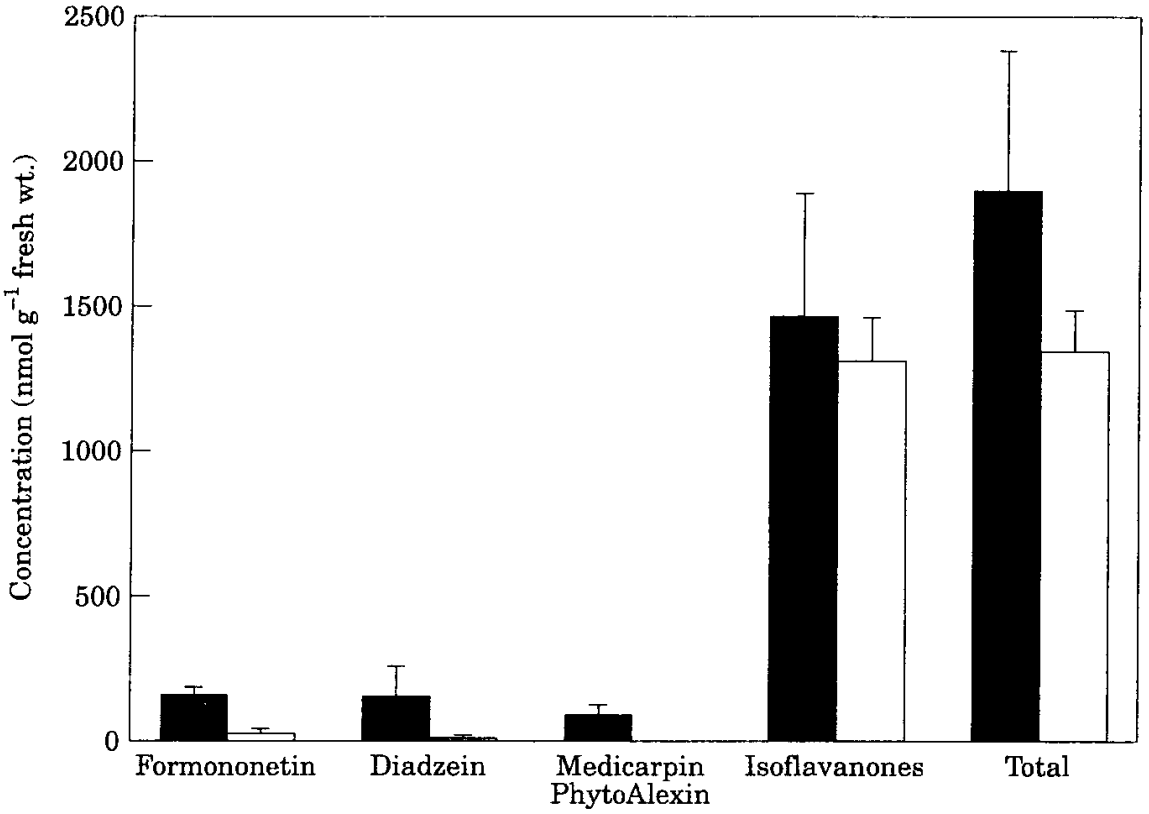

FIG. 4. Comparison of the phytoalexin response of groundnut leaves (cv. TMV2) challenged by the most effective biotic agent, *Cercospora arachidicola*-severe infection (ELSH) with the most effective abiotic treatment, salicylic acid-foliar spray (0·01 M) (SAFS). For constitution of the isoflavanone component see tables 3 and 4. Data represent means of three replicates. Vertical bars are standard deviations. (■) ELSH; (□) SAFS.

DISCUSSION

Several lines of evidence suggest that phytoalexin production may be an important mechanism by which plants resist pathogens. Among these are their accumulation to inhibitory concentrations at the time when the pathogen ceases to grow [*19*], the ability of virulent organisms to degrade their hosts' phytoalexins [*23*] and the fact that transgenic plants expressing a foreign phytoalexin are more resistant [*7*]. One way in which this mechanism may be used to increase resistance of a crop plant is to exploit the natural variation within the species with regard to chemical structures of the phytoalexins elicited, their speed of accumulation and the concentrations attained. However, few studies of such variation have been made [*4*, *11*]. Two reasons for this are the multiple compounds produced and, when they are elicited by a pathogen, the possibility that they may be metabolized by the infecting organism.

Results of the present paper are in agreement with those of Edwards *et al.* [*6*] who found that the necrotrophic pathogen, *C. arachidicola*, elicited the highest concentrations of phytoalexins whereas *P. arachidis*, which is a biotroph, elicited the least (Table 1).

Moreover, these differences were qualitative as well as quantitative, high concentrations of the more polar isoflavanones 1 and 2 being found in extracts of leaves infected with the two necrotrophs (*C. arachidicola* and *P. personata*). This result raises the possibility that the more polar isoflavanones are the products of metabolism of less polar compounds by the fungi. Arnoldi and Merlini [*1*] showed that within groups of isoflavanones of similar structure an increase in lipophilicity correlated positively with increased antifungal activity. Reciprocally, Edwards *et al.* [*6*] suggested that *C. arachidicola* metabolized medicarpin by demethylation to the more polar and less antifungal demethylmedicarpin, metabolism of phytoalexins to more polar compounds being a common method of detoxification by pathogenic fungi [*23*]. Alternatively, the necrotrophs may have influenced the biosynthesis of the phytoalexins towards the production of the more polar compounds. In contrast, isoflavanones 1 and 2 neither accumulated in leaves infected by *P. arachidis* nor in those subjected to abiotic elicitors.

Edwards *et al.* [*6*] found that the pterocarpans, medicarpin and demethylmedicarpin, were the major components of the phytoalexin response in cv. Egret infected with *C. arachidicola* rather than the isoflavanones shown in the present study. This difference may be attributed to genotype, since Egret is a member of the botanical type, *A. hypogaea* ssp. *hypogaea* var. *hypogaea*, whereas TMV2 is of the type *A. hypogaea* ssp. *fastigiata* var. *vulgaris* [*25*].

Phytoalexin elicitation by salicylic acid in whole tissues does not appear to have been reported previously although Kauss *et al.* [*10*] demonstrated that salicylic acid and, to a greater extent, chlorine derivatives of the compound as well as acetyl salicylic acid (aspirin), enhanced phytoalexin induced by sub-optimal levels of a fungal elicitor in cell suspensions of parsley. Unfortunately, salicylic acid was not tested in the absence of the elicitor. In the present experiments abraded but not intact leaves responded to salicylic acid by the accumulation of concentrations of phytoalexins that approached those of infection with *C. arachidicola* and was more than three-fold greater than those found in leaves irradiated abaxially by u.v. Salicylic acid is required for both systemic acquired resistance and genetic resistance in plants [*3*]. One way in which it may operate is through the induction of PR-proteins but there has been little documentation of phytoalexin elicitation by this compound. In our studies, wounding leaves by abrasion with carborundum was necessary for the spray with salicylic acid to be effective, unwounded leaves failing to accumulate any phytoalexin. Spraying abraded leaves with salicylic acid caused greater necrosis than spraying of similar leaves with water. Therefore, salicylic acid appears to be enhancing the reaction of the host to abrasion. This experiment recalls the findings of Hargreaves and Bailey [*8*] and Rahe and Arnold [*16*] who showed in bean hypocotyls that it was the conjunction of live and necrotic cells that caused the accumulation of phytoalexins.

Elicitation of phytoalexins by salicylic acid was faster than infection with fungi and, compared with severe infection by *C. arachidicola*, more reproducible. This suggests that it would be worth extending the study to a range of groundnut cultivars, varying in resistance to leaf-infecting fungi. Such an investigation might show whether spraying abraded leaves with salicylic acid could form the basis of a screening technique for determining the phytoalexin potential of groundnut cultivars.

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REFERENCES

1. **Arnoldi A, Merlini L.** 1990. Lipophilicity-antifungal activity relationships for some isoflavanoid phytoalexins. *Journal of Agricultural Food Chemistry* **38**: 834–838.
2. **Cheong JJ, Hahn MG.** 1991. A specific, high-affinity binding site for the hepta-β-glucoside elicitor exists in soybean membranes. *The Plant Cell* **3**: 137–147.
3. **Delaney T, Uknes S, Vernooij B, Freidrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessman H, Ward E, Ryals J.** 1994. A central role of salicylic acid in plant disease resistance. *Science* **266**: 1247–1250.
4. **Desjardins AE, McCormick SP, Corsini DL.** 1995. Diversity of sesquiterpenes in 46 potato cultivars and breeding selections. *Journal of Agriculture Food and Chemistry* **43**: 2267–2272.
5. **Edwards C, Strange RN.** 1991. Separation 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. *Journal of Chromatography* **547**: 185–193.
6. **Edwards C, Strange RN, Cole DL.** 1995. Accumulation of isoflavanoid phytoalexins in leaves of *Arachis hypogaea* differing in reaction to rust (*Puccinia arachidis*) and early leafspot (*Cercospora arachidicola*). *Plant Pathology* **44**: 573–579.
7. **Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier PH, Stöcker RH, Stenzel K.** 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* **361**: 153–156.
8. **Hargreaves JA, Bailey JA.** 1978. Phytoalexin induction by hypocotyls of *Phaseolus vulgaris* in response to constitutive metabolites released by damaged bean cells. *Physiological Plant Pathology* **13**: 89–100.
9. **Homans AL, Fuchs A.** 1970. Direct bioautography on thin layer chromatograms as a method for detecting fungitoxic substances. *Journal of Chromatography* **51**: 327–329.
10. **Kauss H, Franke R, Krause K, Conrath U, Jeblick W, Grimmig B, Matern U.** 1993. Conditioning of parsley (*Petroselinum crispum* L.) suspension cells increases elicitor-induced incorporation of cell wall phenolics. *Plant Physiology* **102**: 459–466.
11. **Kessmann H, Barz W.** 1987. Accumulation of isoflavones and pterocarpan phytoalexins in cell suspension cultures of different cultivars of chickpea (*Cicer arietinum*). *Plant Cell Reports* **6**: 55–59.
12. **Kleissig DF, Malamy J.** 1994. The salicylic acid signal in plants. *Plant Molecular Biology* **26**: 1439–1458.
13. **Kodama O, Suzuki T, Miyakawa J, Akatsuka T.** 1988. Ultraviolet induced accumulation of phytoalexins in rice leaves. *Agricultural and Biological Chemistry* **52**: 2469–2473.
14. **McDonald D, Subrahmanyam P, Gibbons RW, Smith DH.** 1985. Early and late leaf spots of groundnut. *Information Bulletin no. 21. Patancheru, AP, 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.*
15. **Minton NA, Brenneman TB, Bondari K, Harrison GW.** 1993. Activity of fosthiazate against *Meloidogyne arenaria, Frankliniella* spp., and *Sclerotium rolfsii* in peanut. *Peanut Science* **20**: 66–70.
16. **Rahe JE, Arnold RM.** 1975. Injury-related phaseollin accumulation in *Phaseolus vulgaris* and its implications with regard to specificity of host-parasite interaction. *Canadian Journal of Botany* **53**: 921–928.
17. **Raskin I.** 1992. Role of salicylic acid in plants. *Annual Review of Plant Physiology* **43**: 439–463.
18. **Reddy DVR.** 1984. Tomato spotted wilt virus. In: Morris Porter D, Smith Donald H, Rodríguez-kábana R, eds. *Compendium of Peanut Diseases.* St. Paul, U.S.A.: The American Phytopathological Society, 48–49.
19. **Smith CJ.** 1996. Accumulation of phytoalexins: defence mechanism and stimulus response system. *New Phytologist* **132**: 1–45.
20. **Smith DH, Littrell RH.** 1980. Management of peanut foliar diseases with fungicides. *Plant Diseases* **64**: 356–361.
21. **Subba Rao PV, Strange RN.** 1995. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack. In: Daniel M, Purkayastha RP, eds. *Handbook of Phytoalexin Metabolism and Action.* New York, U.S.A.: Marcel Dekker, Inc, 199–277.
22. **Subrahmanyam P, Williams JH, McDonald D, Gibbons RW.** 1984. The influence of foliar diseases and their control by selective fungicides on a range of groundnut (*Arachis hypogaea* L.) genotypes. *Annals of Applied Biology* **104**: 467–476.
23. **Van Etten HD, Matthews DE, Matthews PS.** 1989. Phytoalexin detoxification: importance for pathogenicity and practical implications. *Annual Review of Phytopathology* **27**: 143–164.

24. **Wadia KDR, Butler DR.** 1994. Relationships between temperature and latent periods of rust and leaf spot diseases of groundnut. *Plant Pathology* **43**: 121–129.
25. **Wynne JC, Coffelt TA.** 1982. Genetics of *Arachis hypogaea* L. In: Pattee HE, Young CT, eds. *Peanut Science and Technology*. Yoakum, U.S.A.: American Peanut Research and Education Society, Inc, 50–94.
26. **Wynne JC, Beute MK, Nigam SN.** 1991. Breeding for disease resistance in peanut (*Arachis hypogaea* L.). *Annual Review of Phytopathology* **29**: 279–303.