New dienol phytoalexins isolated from *Arachis hypogaea* leaves infected with *Puccinia arachidis* Speg.

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Summary. — Two dienol phytoalexins, 9-hydroxy 10,12 octadecadien oic methyl ester and 13-hydroxy 9,11 octadecadien oic methyl ester were isolated from groundnut leaves infected with *Puccinia arachidis*. Extraction, purification characterization and the antifungal activity of these compounds are described and their possible role in host defences against pathogenic infections is discussed.

INTRODUCTION

Accumulation of phytoalexins against natural microflora was widely reported from groundnut seeds [Ahuamah *et al.*, 1981 ; Ingham, 1976 ; Keen, 1975 ; Keen and Ingham, 1976]. Strange *et al.*, [1985] isolated medicarpin from groundnut leaves infected with either *Cercospora arachidicola* or *Phoma arachidis*, both foliar pathogens. We previously showed that methyl linolenate is produced in groundnut leaves infected with *Puccinia arachidis* [Subba Rao *et al.*, 1988]. We now report isolation of two new dienol phytoalexins as efficient fungal inhibitors from rust-infected groundnut leaves.

MATERIALS AND METHODS

Extraction and purification of phytoalexins. Groundnut leaves of a local Spanish variety, from both healthy and infected (with *P. arachidis*) plants were collected from two month-old crop in the farmers' fields. They were later grinded (Sorvall Omni-Mixer 230, USA) in 50 % methanol (MeOH) in water after a thorough wash in distilled water. Extracts were left to diffuse in dark at room-temperature for 48 hr and filtered through No. 1 glass filter. Filtered extracts were concentrated to 1/4 volume using a rotative evaporator (Buchi, Switzerland) at 40 °C and were centrifuged for 10 min. at 12,000 rpm/min. The supernatant was extracted thrice with n-hexane (Hx) and the aqueous phase was further extracted thrice with ethyl acetate (EtOAc). Dienols were detected on TLC plates (0.25 mm thick, Silica Gel 60 F_{254}) using Hx and EtOAc (5 : 1 or 2 : 1) as solvent system. EtOAc phase was concentrated to 5 ml and was chromatographed on an atmospheric silica column using Hx : EtOAc : MeOH (50 : 50 : 5) as eluant system. The fraction containing antifungal compound was later chromatographed on a HPLC column.

Separation by HPLC. Samples (0.2 ml) were injected onto a silica column (30 × 0.63 cm) and were eluted with Hx : EtOAc (65 : 35 v/v) at a flow rate of 2 ml/min. The HPLC detector was set at 280 nm. Active fractions were initially defined by their ability to inhibit *Cladosporium cladosporioides* in a TLC assay [Subba Rao *et al.*, 1988] and subsequently by their Rf and absorption of light at 280 nM. Further purification was achieved by a second HPLC C18 column (30 × 0.63 cm). The compounds were eluted with MeOH : H_2O : CH_3COOH from 50 : 50 : 01 to 99 : 00 : 01 (v/v) in 20 min. with 1 ml/min. flow rate.

Characterization. The active fractions purified as above were subjected to a Gas Chromatograph (GC) (CP Wax capillary column of 25 m long and 0.32 mm internal diameter) — Mass Spectrometer (MS) (Nermag RIO-10C) instrument.

Antifungal assays. The two dienols were assayed for their effects on spore germination and germ tube growth of *P. arachidis in vitro* as previously described (Subba Rao *et al.*, 1988). Dienol concentrations ranging from 0.5 µg/ml to 10 µg/ml were used during the assays. Germ tube length was measured of 100 urediniospores using an occular micrometer and the average values were calculated and compared with those of 'Control' to which the dienols were not added.

RESULTS

Following extraction and separation on TLC as described under materials and methods, a major antifungal component was separated by a reverse phase semi-preparative HPLC. GC-MS analysis indicated the presence of more than one isomer of dienic alcohols. These compounds are related to dimorphecolic or coriolic acid series. Under the GC conditions used during our investigations, the dienols were eluted at 220 °C. Under electron impact (EI), two major isomers were detected and were identified by comparing their retention time and characteristic ions with those of reference compounds (J. M. Duffault, Ph. D., Thesis in preparation). Of these two isomers, of probable cis, trans (-hydroxy) geometry, 9-hydroxy 10,12 octadecadien oic methyl ester was usually predominent in the samples analysed during our investigations.

The first isomer 9-hydroxy 10,12 octadecadien oic methyl ester was characterized by the molecular ion (M = 310) and ions at m/z 292 $(M-18)^+$, 185, 155, 153, 125, 83, 81, 79, 67, 55. The second isomer, 13-hydroxy 9,11 octadecadien oic methyl ester was characterized by the ions at m/z 310 (M^+), 99 (base peak), 83, 81, 79, 71, 67, 55. Confirmatory evidence for the structural determination of these compounds was obtained by chemical ionization (CI)-mass spectrometry using ammonia (NH_3) as reagent gas. Under these conditions characteristic ions observed at m/z 328 $(M + NH_4)^+$ and at m/z 311 (MH^+) demonstrated the molecular weight of these compounds as 310. These results were further

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confirmed using nitric oxide (NO) as reagent gas. Under Ci-No$^+$ conditions, the mass spectra revealed the presence of ions at 340 $(M + NO)^+$ and at 310 $(M)^+$. Other ions produced, *i.e.* for the first compound, at m/z 308 or $(M - 2H)^+$, 292 $(M - H_{éà})^+$, 185 ; 155 were characteristic of the hydroxyl group and of its position in the chain. These results are in agreement with those of others carried out on other host-pathogen interactions (*Lycopersicum esculantum-Phytophthora parasitica ;* Vernenghi *et al.*, 1985 ; *Elaeis guinensis-Fusarium oxysporum* f. sp. *elaeidis ;* Vernenghi *et al.*, 1987). Detection of these new class of phytoalexins in several host-pathogen interactions led to the development of specific mass-spectrometric methods based on gas phase ion-molecular reactions (Einhorn *et al.*, 1988, 1989). This methodology will further lead to the direct localization of the dienol systems for structural determination of new analogs of such phytoalexins or facilitate fast screening of infected plant tissue-extracts.

The dienic alcohols were observed at Rf : 0.87-0.90 on TLC plates in Hx : EtOH : MeOH (50 : 50 : 5 v/v) while they get separated into two bands in Hx : EtoH (5 : 1 v/v) at Rf : 0.48 and 0.52 and at 0.65 and 0.67 in Hx : EtOH (2 : 1 v/v) respectively. They turn dark brown with saturated $SbCl_3$ in $CHCl_3$ when kept at 110 °C for 20 min.

Dienols showed total inhibition of spore germination and germ-tube growth of *P. arachidis* even at a concentration as low as 10 µg/ml. There was no effect on the spore germination of *P. arachidis* urediniospores at much lower concentrations ranging from 0.5 µg/ml to 2 µg/ml. On the other hand, the germ tube growth was affected significantly by the same range of concentrations and was close to 100 % at 5 µg/ml concentration (Table I).

TABLE I. — ***In vitro* antifungal activity of dienols to *Puccinia arachidis* (1)**

Dienol concentration (µg/ml)	Inhibition of spore germination (%)	Germ tube length (mm)
Control	0	0.21
0.5	3	0.18
1.0	4	0.19
2.0	0	0.11
5.0	74	0.03
10.0	100	0.00

(1) Measured after 3 h incubation.

DISCUSSION

Ingham (1976), Keen (1975), Keen and Ingham (1976) showed that groundnut plants produce stilbene phytoalexins. Later Strange *et al.*, (1985) showed that the pterocarpan phytoalexin, 'medicarpin' was also produced by the same host. We showed that groundnut plant could also produce a different group of phytoalexin namely, methyl ester of a polyunsaturated fatty acid, methyl linolenate (Subba Rao *et al.*, 1988). Following our previous work, we now report the isolation of two dienol phytoalexins from groundnut leaves in this paper. Similar compounds was previously isolated from other host-plants such as tomato [Vernenghi *et al.*, 1985] and oil-palm [Vernenghi *et al.*, 1987] against pathogenic infections. There is evidence that the dienic alcohols are efficient inhibitors of fungal growth [Vernenghi *et al.*, 1985]. Differential accumulation of 9-hydroxy and 13-hydroxy octadecadien oic methyl esters were also reported in young oil-palm tissues [Vernenghi *et al.*, 1987] and their accumulation could be modified by the application of different fungal or synthetic elicitors [Subba Rao, 1985]. Considerable degree of synergestic effect in terms of antifungal activity between dienols and the phenolic phytoalexins was reported in oil palm — *Fusarium oxysporum* f. sp. *elaeidis* interactions [Vernenghi *et al.*, 1987]. It is particularly interesting to note that the antifungal activity of these phytoalexins is at least 20 times more than that of methyl linolenate [Subba Rao *et al.*, 1988]. In view of their high antifungal nature, higher concentrations at which they are produced by the groundnut leaf tissues and increasing evidence of their involvement in several host-parasite interactions, studies are needed to establish their role in host resistance, if any and to exploit that information for use in resistance breeding. Studies are in progress to verify the possible production of other phytoalexins by the groundnut plant in interactions with *P. arachidis*.

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RÉSUMÉ

Nouvelles phytoalexines diénoliques isolées à partir de feuilles d'*arachis hypogaea* infestées par *Puccinia arachidis* Speg.

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Deux phytoalexines diénoliques, l'ester méthylique 9-hydroxy 10,12 octadécadiénoïque et l'ester méthylique 13-hydroxy 9,11 octadécadiénoïque, ont été isolées à partir de feuilles d'arachide infestées par *Puccinia arachidis*. L'extraction, la purification, la caractérisation et l'activité anti-fongique de ces composés sont présentées, et leur rôle éventuel dans la protection des hôtes contre l'infestation pathogénique est discuté.

RESUMEN

Nuevas fitoalexinas dienólicas aisladas de hojas de *Arachis hypogaea* infestadas por *Puccinia arachidis* Speg.

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Dos fitoalexinas dienólicas, el éster metílico 9-hidroxi 10,12 octadecadienóico y el éster metílico 13-hidroxi 9,11 octodecadienóico, se aislaron de hojas de maní infestadas por *Puccinia arachidis*. La extracción, la purificación, la caracterización y la actividad antifúngica de estos compuestos se presentan, discutiéndose su posible papel en la protección de los hospederos contra la infestación por patógenos.