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INDUCTION OF PAL ACTIVITY AND DIHYDROSTILBENE PHYTOALEXINS IN *DIOSCOREA ALATA* AND THEIR PLANT GROWTH INHIBITORY PROPERTIES

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Key Word Index—*Dioscorea alata*; Dioscoreaceae; *Botryodiplodia theobromae*; *Sorghum bicolor*; Gramineae; *Cladosporium cladosporoides*; water yam; dihydrostilbene phytoalexins; phenylalanine ammonia lyase; growth inhibition

Abstract—Dihydropinosylvin, batatasin IV, demethylbatatasin IV and batatasin III were found in the water yam (*Dioscorea alata*) which had been inoculated with *Botryodiplodia theobromae* or treated with mercuric chloride. Following induction, transient increases were observed in the first three compounds and this was preceded by a transient increase in the activity of phenylalanine ammonia lyase but not tyrosine ammonia lyase activity. In mercuric chloride treated tubers an increase in polyphenol oxidase was also observed. The dormancy inducing compounds dihydropinosylvin and batatasin IV were also found to inhibit the germination of seeds of and root elongation in young seedlings of *Sorghum bicolor*. By comparison, demethylbatatasin IV was not inhibitory.

INTRODUCTION

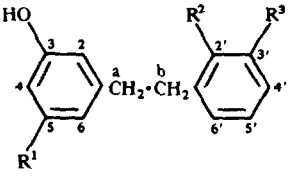
The water yam, *Dioscorea alata* (Dioscoreaceae, Section Enantiophyllum) is grown extensively as a food in West Africa [1]. In response to *Pseudomonas* infection, *D. opposita* Thumb. (*D. batatas* Decne) produces dihydropinosylvin (**1**) and *D. rotundata* Poir. produces **1**, batatasin IV (**3**) and demethylbatatasin IV (**2**) as phytoalexins [2, 3]. These compounds, which may be produced *de novo* or show increased production on elicitation, together with batatasins I–V have been implicated as dormancy inducing compounds in *D. opposita* [4, 3]. Some of these dihydrostilbenes also have plant growth inhibitory properties [5, 6]. As all of these compounds are phenanthrene and stilbene analogues it has been proposed that they are products of phenylalanine metabolism, [7, 8].

In continuation of our studies on fungitoxic compounds isolated from *Dioscorea* species, [9], we have isolated four dihydrostilbene phytoalexins from *D. alata* and have studied their rates of accumulation in fungal or mercuric chloride ($HgCl_2$) treated yams in relation to the activities of phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) and polyphenol oxidase (PPO). In conjunction with this study the growth inhibitory properties of **1** and **2** have been compared with **3**, a known inhibitor of plant growth [6].

RESULTS AND DISCUSSION

Isolation of dihydrostilbenes

Extraction of *D. alata* tubers infected with *Botryodiplodia theobromae*, with separation and purification accomplished using the techniques given in Experimental, gave five compounds four of which were readily identified as dihydropinosylvin (**1**), demethyl batatasin IV (**2**), batatasin IV (**3**) and batatasin III (**4**) by comparison of their spectral data with those of authentic samples and literature values, [2, 5, 9]. A fifth compound, A_1, was obtained in an amount too small to allow for structural elucidation. It gave a blue colour, similar to batatasin I, with vanillin in sulphuric acid, but differed in R_f value when subjected to co-chromatography with batatasin I in various solvent systems.



1 R^1 = OH, R^2 = R^3 = H
2 R^1 = OH, R^2 = OH, R^3 = H
3 R^1 = OMe, R^2 = OH, R^3 = H
4 R^1 = OMe, R^3 = OH, R^2 = H

In contrast to the report of Ireland *et al.* [10] we were unable to locate batatasin I in *D. alata* tubers either prior to or subsequent to elicitation with *B. theobromae*, $HgCl_2$ or sterile water. Control samples of *D. alata* did not yield these dihydrostilbenes.

Rate of accumulation of dihydrostilbenes in D. alata *tubers in response to* B. theobromae *or* $HgCl_2$

Figures 1a and 2a show that **1**–**3** accumulate in yam tubers infected with *B. theobromae* in contrast to yam

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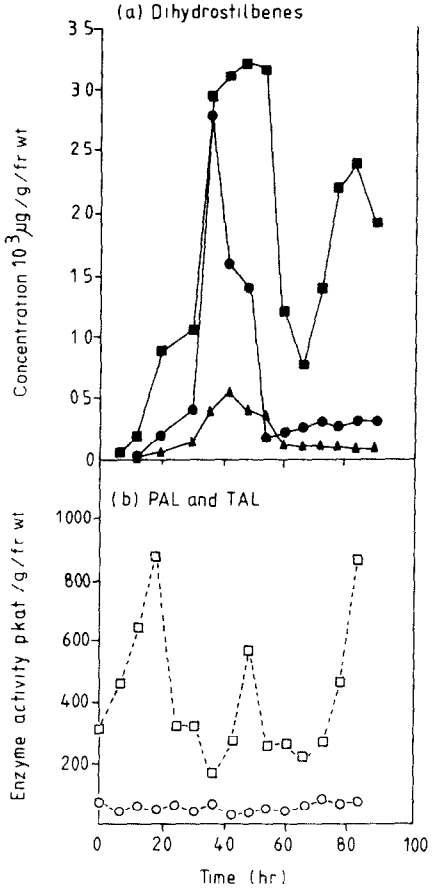

Fig. 1 Enzyme activities in relation to the accumulation of dihydrostilbenes 1–3 in *B theobromae* induced tubers ■—■ dihydropinosylvin (1), ●—● demethylbatatasin IV (2), ▲—▲ batatasin IV (3), □—□ phenylalanine ammonia lyase (PAL), ○—○ tyrosine ammonia lyase (TAL)

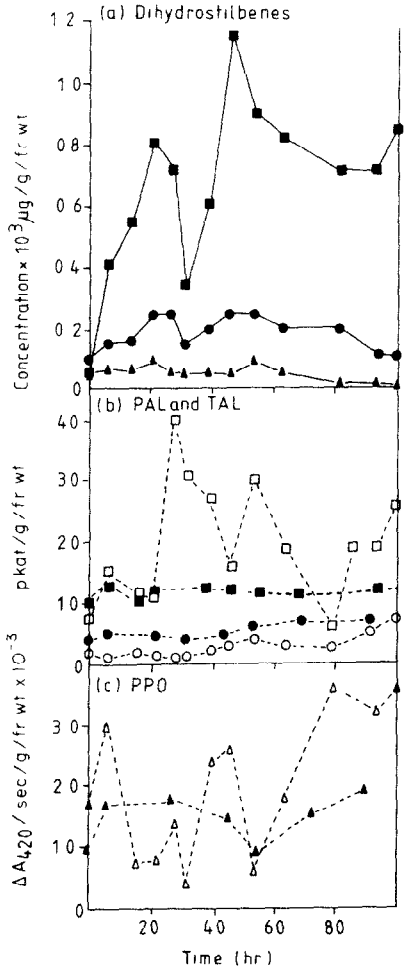

Fig 2 Enzyme activities in relation to the accumulation of dihydrostilbenes 1–3 in $HgCl_2$ induced tubers (a) ■—■ dihydropinosylvin (1), ●—● demethylbatatasin IV (2), ▲—▲ batatasin IV (3). (b) After elicitation □--□ PAL, ○--○ TAL, in controls ■—■ PAL, ●—● TAL. (c) After elicitation △—△ PPO, in controls ▲—▲ PPO

tubers treated with $HgCl_2$ where only 1 shows appreciable increases in response to treatment In neither case did the compounds show any appreciable accumulation in the controls until after 72 hr and 1 never rose beyond 380 µg/g fr. wt Although large amounts of 1 and 2 were produced in response to *B. theobromae* [3.2 mg/g fr. wt after 50 hr (1) and 2 8 mg/g fr wt after 30 hr (2)] the accumulation of 1 in response to $HgCl_2$ was much lower (1.16 mg/g fr wt after 50 hr) as was the accumulation of 2 and 3 In both experiments amounts of 1, and for the *B. theobromae* experiment 2, varied over the 100 hr period of the experiment suggesting not only active formation of these compounds but also rapid metabolism It has been suggested that the biosynthesis of phenanthrenes and dihydrophenanthrenes proceeds via dihydro-*m*-coumaric acid in the Combretaceae and Dioscoraceae. The dihydrostilbenes, therefore, arise via this route to give demethylbatatasin IV which is subsequently metabolized to batatasin IV in *Dioscorea* [11] However, the position of dihydropinosylvin remains unclear as this could be produced either via a parallel pathway or via the loss of a hydroxyl from demethylbatatasin IV In the current experiments activation of the enzymes of the pathway to the dihydrostilbenes is much more effective using *B theobromae* as an elicitor and the present results would suggest that some of the enzymes of phenylpropanoid metabolism important in stilbene formation are activated in response to elicitation by either fungi or inorganic ions

Activities of PAL, TAL and PPO during dihydrostilbene accumulation

The simultaneous assay of PAL, TAL and PPO during the accumulation of dihydrostilbenes after elicitation with *B theobromae* or $HgCl_2$ produced the following results As shown in Fig 1b, PAL activity appeared to precede the accumulation of dihydrostilbenes in experiments using *B theobromae* as an elicitor and a similar effect was observed in experiments utilizing $HgCl_2$ as the elicitor (Fig 2b) In each case, the increase in enzyme activity precedes the onset of dihydrostilbene formation. However, in the case of $HgCl_2$ elicitation the levels of PAL were a fraction (0.46%) of those produced using *B theobromae* as an elicitor and TAL activity was somewhat lower than that in the controls which may relate to

some inhibition of these enzymes (PAL and TAL) by $HgCl_2$. The transient increases in PAL and TAL activity and dihydrostilbene formation with elicitation by *B. theobromae* were not observed in the control samples of tuber where levels of PAL and TAL were *ca* 10–20 and 5–10 pkat/g fr. wt (Fig. 2b)

The results of elicitation with *B. theobromae* suggest the cellular synthesis of the dihydrostilbenes from phenylalanine rather than tyrosine since TAL activity was present at low levels compared with PAL with both elicitors (Figs 1b and 2b) and it is not stimulated as compared with the controls. However, the concomitant increase in production of **1** and **2** in tubers infected with *B. theobromae* after 30–60 hr and the later second surge in production of **1** without production of **2** suggests that either **2** is not formed directly from **1** or that the required hydroxylating enzyme is activated for a much shorter period of time than are the enzymes leading to the formation of **1**. A third possibility is that enzymes which lead to the breakdown of **2** and **3** may be activated and, therefore, their long term accumulation will not occur

The overall amounts of **1** produced by $HgCl_2$ treatment of tubers was much lower than that with *B. theobromae* elicitation, a factor which may relate directly to enzyme inhibition and also to the rapid onset of cell death [12, 13] observed in experiments using $HgCl_2$ Nonetheless, the suggestion [14] that abiotic inducers do not affect phytoalexin synthesis but only inhibit their degradation would not appear to be supported by the present results (Fig. 2a, b)

PPO activity (Fig. 2c) monitored in experiments with controls and $HgCl_2$ induced tubers did not show any correlation with the progressive browning which was observed in these experiments. Its transient increase in $HgCl_2$ induced tubers supports the proposal for an altered role for PPO in stressed systems [15, 16] where its availability may assist in phenolic deposition in cell walls as a mechanism to limit infections.

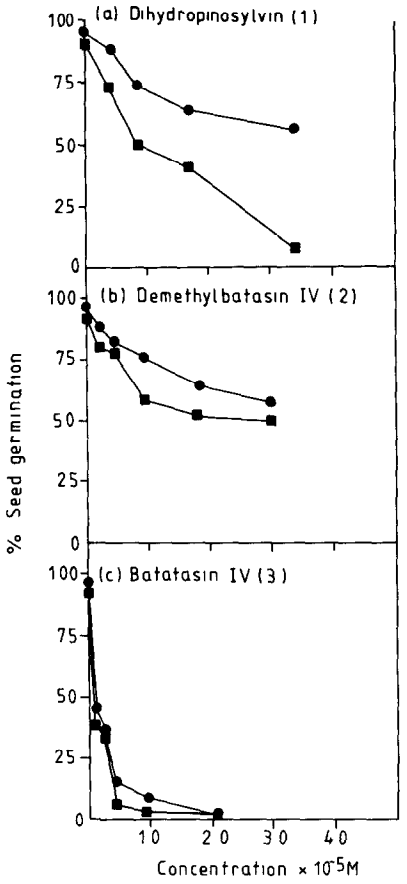

Fig 3 Inhibitory activites of dihydrostilbenes **1–3** on germination of *S bicolor* seeds. ●—● 48 hr incubation, ■—■ 24 hr incubation

Growth inhibitory activity

The growth inhibitory activities of batatasin IV (**3**) and batatasin III (**4**) in lettuce seed germination, hypocotyl elongation and wheat coleoptile section elongation tests have been established [6]. In our experiments investigating the effect of dihydrostilbenes on seed germination using *Sorghum bicolor*, batatasin IV (**3**) showed over 90% inhibition of germination (Fig. 3c) at both 24 and 48 hr from commencement of treatment. Dihydropinosylvin (**1**) shows similar activity at 10^{-5}M after 24 hr but the seeds were observed to largely recover after 48 hr incubation (Fig. 3a) Demethylbatatasin IV (**2**) did not appear to have as appreciable an effect on seed germination (Fig. 3b) as did **1** and **3**, maximum inhibition (at 24 hr) was *ca* 40%. Although not presented here, similar results were observed when *Zea mays* seeds were used for germination tests.

The growth inhibitory effect of **1** (Figs 3a and 4a) and **2** (Figs 3b and 4b) were compared to that of their structural analogue **3** (Figs 3c and 4c) using seedlings of *S bicolor*. Batatasin IV (**3**) exhibited the expected strong inhibition of *S. bicolor* root elongation (Fig. 4c) and **1** was found to exert a similar effect Demethylbatatasin IV (**2**) was, however, somewhat less effective than the other two compounds but was still an effective inhibitor of root elongation. In all experiments roots did not recover with time and root tips, at high concentrations of **1** and **3**, collapsed presumably due to an uncontrolled leakage of metabolites and electrolytes resulting from the severe effects of these compounds on cell membranes [17].

While it might be expected that compounds with reduced polarity due to fewer hydroxyl groups (**1**) or methylation (**3**) may cross the cell membrane with greater ease this is not totally reflected in the above results. All three compounds appear to be phytotoxic, although, in the seed germination test, inhibition of germination after 48 hr was much less well defined with **1** and **2** with 60–70% of all seeds germinating (Fig. 3).

Our results with both tests suggest that a multiplicity of effects on cell membranes and cell biochemistry are involved in the inhibition of seed germination and seedling growth by dihydrostilbenes

EXPERIMENTAL

Procedures for the isolation and identification of the dihydrostilbenes **1–4** were described earlier [3] Compounds **1–3** were authenticated by comparison with UV, MS, and ^{1}H NMR data given in refs [2, 9], **4** was authenticated using data given in refs [3, 4].

Materials. L-Phenylalanine, L-tyrosine, cinnamic acid and *p*-hydroxycinnamic acid were obtained from Sigma (London).

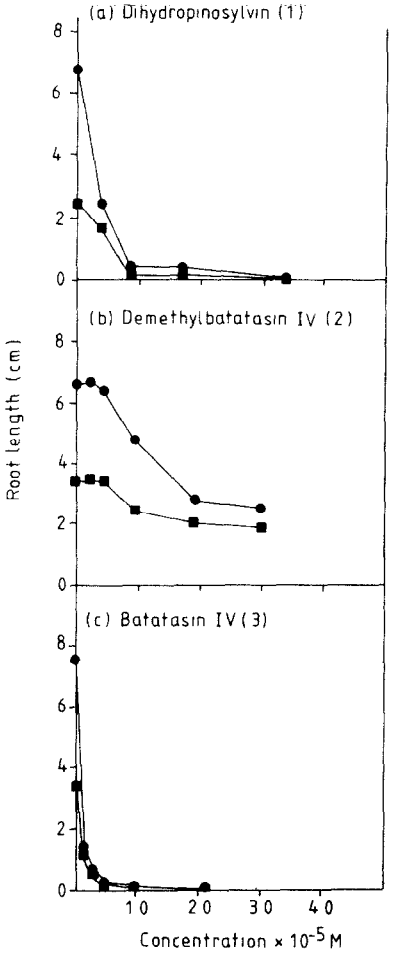

Fig 4 Inhibitory activities of dihydrostilbenes **1–3** on root elongation of *S bicolor* seedlings ● –● 48 hr incubation, ■— ■ 24 hr incubation

Samples of batatasin I and batatasin III were provided by Professor T Hashimoto (Faculty of Science, Kobe University, Kobe, Japan) Tubers of *D alata* L and seeds of *S bicolor* (L.) Mench were purchased in the market in Ile-Ife, Oyo State, Nigeria

Induction and isolation of dihydrostilbenes Tubers (13 kg) of *D alata* were washed with dist H_2O, the flesh cut into 8–10 mm thick slices and further rinsed with sterile dist H_2O Phytoalexins were induced by dipping tuber slices into a suspension of *B theobromae* Pat mycelia for 10 min before incubation in sterile petri dishes for 72 hr [9] Controls were similarly treated except that dist H_2O was substituted for the *B theobromae* suspension

Tuber slices were extracted with EtOAc for 24 hr, the resulting marc further extracted (× 2) and the pooled EtOAc frs reduced to dryness *in vacuo* at 40° A TLC bioassay using *Cladosporium cladosporoides*, described in refs [3, 18], was used to detect antifungal compounds The crude extract of the induced sample (7 g), containing antifungal activity, was further fractionated by CC on activated charcoal, eluted with successive portions of EtOAc, EtOAc–MeOH (2 1), EtOAc–MeOH (1 2) and MeOH to give frs F_1–F_4 Fractions F_2–F_4 had antifungal activity as measured using the *C cladosporoides* TLC bioassay

Fraction F_2 was subjected to CC using silica gel (mesh 60–120) and an elution programme which consisted of 500 ml each of hexane–$CHCl_3$ (9 1), $CHCl_3$, $CHCl_3$–MeOH (9 1) followed by further increases in MeOH concn to 100% Frs (25 ml) were collected and monitored by TLC [3] Fractions 15–64 yielded three compounds **1**, **3** and a compound, A_1 as well as an oil Frs 76–92 gave **2**

Fraction F_3, chromatographed in a similar manner on silica gel yielded further quantities of **1**–**3**, and also **4** F_4, treated in the same way, yielded **4** alone

Compounds **1–4** were further purified by TLC [9] and identified according to data given in refs [2–4, 9] and by comparison with authentic compounds The yields obtained were μg/g fr wt **1** 250, **2** 130, **3** 115 and **4** 14 6

Time course studies for production of dihydrostilbenes and determination of PAL, TAL and PPO activities Tuber pieces from *D. alata* were divided into three groups One group was inoculated with *B theobromae* following the method described above The second group was soaked in an aq soln of $HgCl_2$ (3 mM) for 5 min and the third was treated with sterile H_2O All were then incubated in petri dishes and samples taken at intervals over a period of 100 hr

Assay for dihydrostilbenes Triplicate samples of yam tissue (40 g) were taken from each group described above over a period of 100 hr (Figs 1a and 2a) and macerated in and extracted with 150 ml of EtOAc for 24 hr The EtOAc was removed under vacuum at 40° and the extract subjected to prep TLC ($CHCl_3$–MeOH, 19 1) together with **1**–**3** as ref markers Bands corresponding to **1**–**3** were scraped off the plates, the compounds eluted from the silica gel with MeOH and the extracts evapd to dryness The residues were dissolved in 5 ml of EtOH and the *A* at 282 nm (**1**), 272 nm (**2**), and 275 nm (**3**) determined Using standard calibration procedures it was possible to calculate the amounts of these compounds over the period of incubation of the yam slices which had been treated with *B theobromae*, $HgCl_2$, or sterile H_2O

Assay for PAL and TAL Triplicate samples of yam tissue (20 g) were taken over a period of 100 hr. They were homogenized in 100 ml of 0 05 M borate buffer, pH 8 6, at 0° and filtered through glass wool The filtrates were centrifuged at 6 000 *g* for 20 min at 4° and the supernatant used as the enzyme source for the assay for PAL and TAL These assays were performed using the procedure described in ref [19] and an incubation time of 2 hr The amount of cinnamic acid and *p*-hydroxycinnamic acid formed from phenylalanine and tyrosine, respectively, was determined spectroscopically from calibration curves of the pure compounds [20]

Assay for PPO Yam tissue (triplicate samples of 1 g) was taken for assay of samples incubated over a period of 100 hr (Fig 2c) and homogenised with 0 2 M K-Pi buffer (pH 6), filtered and centrifuged at 28 000 *g* and the supernatant used as the enzyme source Enzyme assays were performed immediately using the method described in ref [21] using K-Pi buffer (pH 6) and 0 05 M catechol as substrate Activity is expressed as change in *A* per sec per mg fr tuber

Seed germination test Seeds of *S bicolor* were surface sterilized by soaking in 95% EtOH for 1 min before thorough rinsing with 3 changes of sterile dist H_2O Seeds (20) were distributed into petri dishes containing filter paper Different concns of the test compounds were prepd in 50 ml of dist H_2O containing one drop of Tween 20 and sterilized by membrane (Millipore) filtration Control solns were prepd from dist H_2O containing one drop of Tween 20 A 5 ml portion of each concn of each compound were aseptically added to a petri dish before incubation at 27° in the dark The number of seeds that germinated in each dish was counted after 24 and 48 hr of incubation Expts

were performed in quadruplicate and the average per cent germination calculated.

Seedling root elongation test. Seedlings of *S bicolor* which had been grown in sterile H_2O were selected with a root length of between 1 and 2 cm and distributed in petri dishes (20 seedlings in each) The increase in root length of all the seedlings in each dish was measured. The average of readings from 4 dishes per concentration was calculated.

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