Journal of Chromatography, 387 (1987) 536–540
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 161

Note

Rapid, sensitive high-performance liquid chromatographic assay for isoflavonoids from cowpea (*Vigna unguiculata*)

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(Received October 14th, 1986)

Without the aid of high-performance liquid chromatography (HPLC), the assay of isoflavonoid phytoalexins from plant extracts is a lengthy and rather cumbersome task. Using the conventional techniques of solvent extraction followed by thin-layer chromatography (TLC) or column chromatography, previous investigators have incurred losses of up to 35%[1]. Such large losses, due to both inefficient extraction and the inherent lability of most isoflavonoid phytoalexins, severely limit the interpretation of results and hinder an accurate determination of the role played by these compounds in the plant disease-resistance response.

HPLC is well suited to the task of rapid, accurate separation and quantification of isoflavonoid phytoalexins from plant extracts[2–6]. The present assay involves minimal tissue preparation and allows the accurate measurement of phytoalexin concentrations in small amounts of tissue within 40 min of harvest.

The host-pathogen system for which this method was designed is cowpea (*Vigna unguiculata* L. Walp.) infected with specific pathogenic races of *Phytophthora* spp. or tobacco necrosis virus (TNV). To demonstrate this method a report is given of the quantification of isoflavonoid phytoalexins in Red Caloona cowpeas following infection with TNV; Caloona and Poona cowpeas following inoculation with *Phytophthora vignae* Purss., race 2 (PvR2); and Poona cowpeas treated with the anti-oomycete chemical Fosetyl-Al, and then inoculated with *Phytophthora cryptogea* Pethybr., Laff.

MATERIALS AND METHODS

Growth and infection of cowpeas

Cowpea cultivars Red Caloona, Caloona and Poona grown in sand/vermiculite at 28°/20°C and 15 h daylength, were inoculated when 7 days old. Following a modification of the methods of Coutts[7], Red Caloona stems were inoculated with a macerate of TNV-infected French bean (*Phaseolus vulgaris* L.) stem. After spraying with water, the seedlings were incubated in a glasshouse for 3–4 days until symptomatic bronze necrotic streaks appeared. For fungal inoculation, Caloona and Poona seedlings were inoculated with PvR2 or *P. cryptogea* by the cut-stem method of Pochard *et al.*[8]. The seedlings were incubated for 48 h at 28°C in a water saturated atmosphere.

Where indicated, Fosetyl-Al was applied as 100 ml per pot soil drench of a 100 $\mu g\ ml^{-1}$ solution of Aliette, kindly supplied by May and Baker (Melbourne, Australia).

0021-9673/87/$03.50

Calibration

Kievitone, phaseollidin and phaseollin were isolated from TNV-infected French bean hypocotyls according to the methods described by Sutton and Deverall[9] and Cloud[10], and characterised by their R_F values on TLC silica gel plates (Merck 60 F_{254}); chromogenic reactions with *p*-nitroaniline and Fast Blue B salt; UV spectrophotometry; and co-chromatography on HPLC with known standards. Concentrations of kievitone[11], phaseollin[12], vignafuran[13] and phaseollidin[14] were estimated by reference to published molar extinction coefficients.

Standard solutions were made up in the extraction solvent containing the internal standard, anisole (methyl phenyl ether; Synchemica).

Sample preparation

For each analysis approximately 50 mg fresh weight of tissue (*ca.* 2 cm of stem tissue) was macerated in a chilled Tenbroeck tissue grinder with 1 ml of chilled extraction solvent [0.0025% (v/v) anisole in 85% methanol]. The resulting macerate was then filtered through a Millipore HVLP 0.45-μm filter and injected into the HPLC system.

Chromatography

Isoflavonoids were separated on a Hewlett-Packard HPLC system consisting of a Model 1084 B liquid chromatograph; a Model 79875 A spectrophotometric detector monitoring absorption at 280 nm; and a Model 79850 B LC terminal which served as a gradient controller and data station. Samples 10–20 μl were injected via a Rheodyne Model 7125 sample injector and separated on a Si 100 Polyol RP-18, 250 × 4.6 mm I.D., 5 μm column (Serva) coupled with an RP-18 guard column (Brownlee Labs.). Mobile phase was delivered at a flow-rate of 1 ml min^{-1} according to the solvent gradient indicated in Fig. 1a. During analysis both the solvents and column compartment were maintained at 40°C. All the solvents used were HPLC-

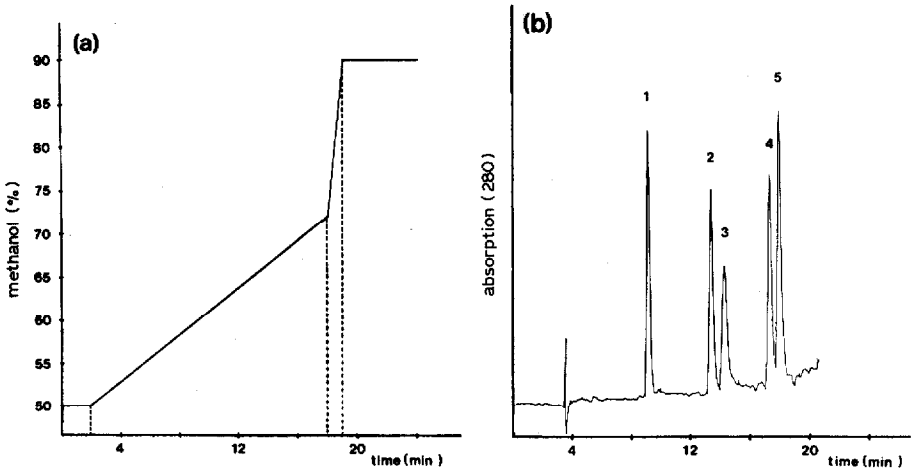

Fig. 1. (a) Methanol–water–acetic acid gradient achieved by running 90% methanol + 1% (v/v) 0.1 *M* acetic acid against 100% water. (b) Gradient elution of a mixture of the internal standard and cowpea phytoalexins. Peaks: 1 = anisole; 2 = phaseollidin; 3 = kievitone; 4 = vignafuran; 5 = phaseollin.

grade (Ajax Chemicals) except water. Distilled water was deionized and filtered through a Milli-Q water purification system. Periodically, the column was flushed with solvents of higher eluting strength, such as 100% methanol or tetrahydrofuran, to minimize ageing effects such as shortening of retention times asnd loss of resolution.

RESULTS

The chromatogram shown in Fig. 1b is a typical calibration run of a mixture of internal and external standards.

A comparison of susceptible (Poona) and resistant (Caloona) cowpea cultivars upon infection with PvR2 is shown in Fig. 2. The response in Caloona tissue after 46 h, was the production of kievitone (peak 3) at a concentration of 186.0 $\mu g\ g^{-1}$ fresh weight. Under identical conditions no detectable levels of kievitone were found in extracts from Poona tissues. Macroscopically, these events correspond with the appearance of limited necrotic lesion in Caloona, and extensive stem rot in Poona.

Fig. 3a shows a chromatogram of a portion of Red Caloona cowpea stem infected with TNV. Where uninfected material produced no response, infected tissue showed the presence of vignafuran (peak 4) at a concentration of 19.2 $\mu g\ g^{-1}$ fresh weight. The chromatogram in Fig. 3b is taken from an assay of Poona cowpeas treated with Fosetyl-Al 24 h before infection with *P. cryptogea*. After 96 h the cowpea responded by accumulating 47.5 $\mu g\ g^{-1}$ fresh weight phaseollidin (peak 2), 956 $\mu g\ g^{-1}$ fresh weight kievitone (peak 3) and 19.5 $\mu g\ g^{-1}$ fresh weight vignafuran (peak 4).

DISCUSSION

The solvent gradient reported here gives satisfactory resolution of the cowpea isoflavonoids kievitone, vignafuran, phaseollidin and phaseollin. The sensitivity of

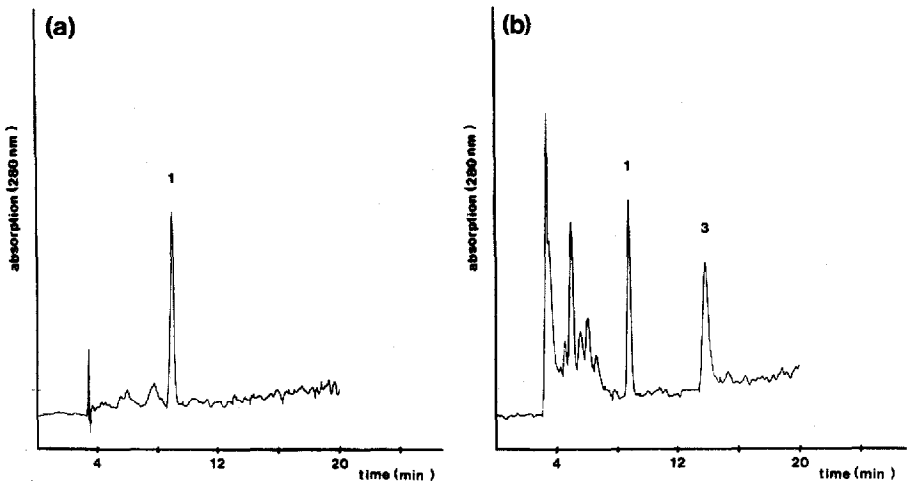

Fig. 2. Chromatograms of extracts of (a) Poona, and (b) Caloona cowpea stems, 46 h after inoculation with PvR2. Peaks numbered as in Fig. 1b.

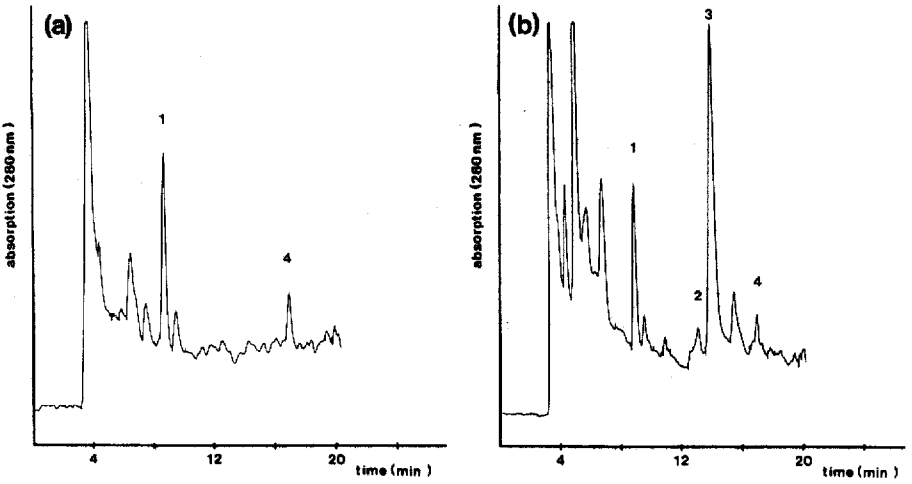

Fig. 3. Chromatograms of extracts of (a) the epicotyl of a Red Caloona cowpea, 96 h after inoculation of the stem with TNV; and (b) the hypocotyl of a Poona cowpea treated with a 100 mg l^{-1} Fosetyl-Al soil drench 24 h before infection with *P. cryptogea* and harvested 96 h after inoculation. Peaks numbered as in Fig. 1b.

the assay allows determinations of just 20 ng of these isoflavonoids from crude macerates of as little as 20 mg fresh weight of tissue; and this rapid assaying of such small amounts of tissue enables the assignation of a role for these isoflavonoids as phytoalexins, since they are known to accumulate only short distances from the inoculation site[15]. Using the same tissue preparation, and the required external calibration, we have also successfully assayed isoflavonoids from cowpea leaves and the hypocotyls of French beans.

Previous analytical HPLC techniques have required much larger tissue samples and more extensive pre-analysis extract processing, which limit their usefulness in interpreting the localized phytoalexin concentrations faced by the invading pathogen. In particular, degradation of the isoflavonoids is minimized by the swiftness of the assay, which allows analysis almost immediately after harvest and the subsequent simultaneous determination of the concentrations of numerous phytoalexins in plant tissue within 40 min.

ACKNOWLEDGEMENTS

This project recieved support from the Australian Research Grants Scheme. We wish to thank Tadeusz Golebiowski for his technical advice.

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