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A convenient, sensitive and rapid assay for antibacterial activity of phytoalexins

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Abstract

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The antibacterial activity of isoflavonoid and sesquiterpenoid phytoalexins was assayed on thin layer chromatography (TLC) plates using *Pseudomonas syringae* pv. *phaseolicola* as the indicator organism in the presence of 2,3,5-triphenyl-tetrazolium chloride (TZC). Addition of glycerol to the overlay nutrient medium, as a carbon source and ultimate electron donor, facilitated the reduction of TZC to pink coloured formazans by bacterial dehydrogenases. Where bacterial growth was inhibited a colourless zone was apparent against a pink background. The test, a modified version of published protocols, gave clear, reproducible results within 24 h.

Key words: Isoflavonoid, sesquiterpenoid, phytoalexin, antibacterial activity, TLC assay

Introduction

There has been a series of publications referring to the antibacterial activity of phytoalexins (Fett & Osman 1982, Gnanamanickam & Patil 1977, Gnanamanickam & Smith 1980, Lyon & Wood 1975, Platero Sanz 1981). Comparison of the results (see Table 1) shows that different authors sometimes came to diverse conclusions as to the activity of any given substance against *Pseudomonas syringae* pv. *phaseolicola*. The authors used a variety of different procedures to assay the antibacterial activity of the substances under test and this probably led to the observed inconsistencies. Indeed, this is apparent from a single report (Fett & Osman 1982) where *P. s.* pv. *phaseolicola* was of intermediate sensitivity to glyceollin in a peptone-water bioassay, but was insensitive in direct spotting and filterpaper disk bioassays. A major difficulty encountered in assaying phytoalexin activity is the high degree of insolubility in aqueous solvents of the compounds being assayed. To circumvent this problem some assay procedures use substances such as dimethylsulphoxide (DMSO) or ethanol as solubilising agents to facilitate

application of phytoalexins to bacteria-seeded agar or liquid medium (Wyman & VanEtten 1978). However, the usefulness of such procedures is limited by the toxicity of the solubilising agents (Fett & Osman 1982).

The method reported here is a modification of that described by Lyon and Wood (1975) who used a nutrient medium without any additional carbon source. The original method described by these authors gave inconsistent and often indistinct results in our hands. *P. s.* pv. *phaseolicola* grows well with glycerol as a carbon source, and when we incorporated this into the overlay medium, colour development was greatly enhanced. Addition of agar as a solidifying agent and an even pouring technique ensured uniform colour development over the surface of the TLC plate except where bacterial growth was inhibited. These modifications to the original procedure facilitated rapid and reliable testing of the antibacterial activity of suspected phytoalexins. The assay is used routinely in our undergraduate practical classes. A TLC procedure for detecting antibacterial activity of phytoalexins has been reported by Wyman & VanEtten (1982). However, their procedure does not include a colour indicator and relies on a visual assessment of bacterial growth against the background of the TLC plate.

Materials and methods

Crude plant extracts or preparations of purified phytoalexins were applied to TLC plates (Silica gel G60 plates poured by ourselves or purchased from Merck) and developed in one of several solvent systems (e.g. chloroform:methanol 33:1, toluene:ethyl acetate:methanol 25:8:1, benzene:acetone 1:1, ethyl acetate:propan-2-ol 9:1). Developed plates were left in a fume cupboard for a least two hours to ensure that all solvents had evaporated before overlaying with bacteria.

Overnight cultures of the indicator strains (in this case various isolates of *P. s.* pv. *phaseolicola* races 1 or 2) were grown in 25 ml Nutrient Broth (Oxoid) supplemented with 1% (v/v) glycerol in a 250 ml Erlenmeyer flask on an orbital shaker at 25–28 °C and 150–200 cycles min^{-1}. King's B broth (King et al. 1954) has also been used successfully to culture the indicator strains, and the culture medium used to grow the indicator cells appears not to be critical for this assay.

The overlay medium for a single TLC plate consisted of 50 ml Nutrient Broth (Oxoid) supplemented with 1% (v/v) glycerol and made 0.6–1.5% (w/v) with Bacto-Agar (Difco), autoclaved at 121 °C for 15 min, cooled in a water bath to 45 °C with 2,3,5-triphenyltetrazolium chloride (Sigma) added to 0.1% (w/v) at this stage. Bacterial cells were collected by centrifugation at 3000 g for 10 min at room temperature and the pellet resuspended in 2 ml buffer (1.4 mM KH_2PO_4, 2.5 mM Na_2HPO_4, pH 7.0). The bacterial suspension was then mixed with 50 ml of the overlay medium and applied *immediately* to the TLC plate. The bacterial suspension was taken up in a 25 or 50 ml pre-warmed pipette and applied evenly over the levelled TLC plate (Fig. 1). It is very important to minimize the time *P. s.* pv. *phaseolicola* cells are kept at 45 °C; since prolonged heat shock can lead to reduced colour development. An alternative to the pipetting procedure is to pour the suspension directly onto the plate. Direct pouring facilitates rapid cooling but can dislodge silica gel, particularly from home-poured plates, and can lead to irregular colour development due to uneven thickness of the overlay. The most even colour development was with relatively high concentrations of agar (1.0–1.5%, w/v) but with some agars, premature geling in the pipette necessitated their use at less than 1%.

After the medium had solidified the overlayed TLC plate was placed in a box lined with moist tissue paper, and incubated overnight at room temperature. Where bacterial growth had been inhibited pale spots could be seen against a deep pink-red background (Fig. 2).

Results

Phytoalexins were not elicited in bean leaves inoculated with killed bacteria (Fig. 2a, lanes 1 & 2) whereas living, avirulent bacteria induced the accumulation of antimicrobial

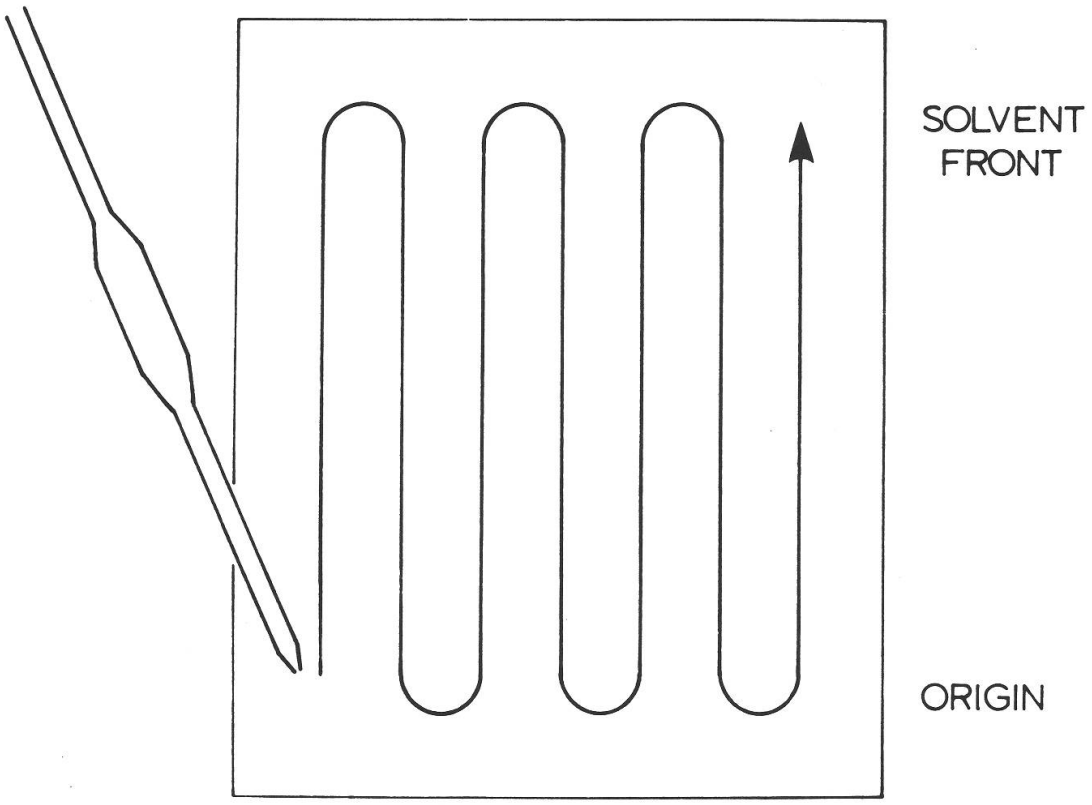

Fig. 1. Overlaying the TLC plate in this manner ensured an even thickness of bacterial suspension and gave uniform background colour development.

Table 1. Inhibition of *Pseudomonas syringae* pv. *phaseolicola* by isoflavonoid and sesquiterpenoid phytoalexins in different assay procedures.

Phytoalexin	Inhibitory	Non-inhibitory
Coumestrol	2, 5*	1, 7
Kievitone	2, 7	3
Phaseollin	2	3, 5, 7
Rishitin	–	6

* 1, Fett & Osman 1982; 2, Gnanamanickam & Patil 1977; 3, Gnanamanickam & Smith 1980; 5, Lyon & Wood 1975; Platero Sanz 1981; 7, Wyman & VanEtten 1978.

Table 2. Minimum inhibitory concentrations of isoflavonoid phytoalexins from French bean in the TLC assay described in this communication. Phytoalexin dissolved in ethyl acetate (0.15 ml) was spotted onto the TLC plate and after development spots of *c.* 2 cm diameter (*c.* 3 cm^2) were present on the plates.

Phytoalexin	Concentration of solution applied ($\mu g\ ml^{-1}$)	Amount in 3 cm^2 spot (μg)
Kievitone	0.3	0.05
Coumestrol	2.0	0.3
Phaseollin	10.0	1.5

substances (Fig. 2a, lane 3). This result shows clearly that fairly crude plant extracts can be assayed without too much apparent interfernce from plant pigments or other endogenous substances. Purified samples of the isoflavonoid phytoalexins phaseollin, coumestrol and kievitone showed antibacterial activity in the assay down to minimum inhibitory doses of 1.5, 0.3 and 0.05 µg respectively (Table 2).

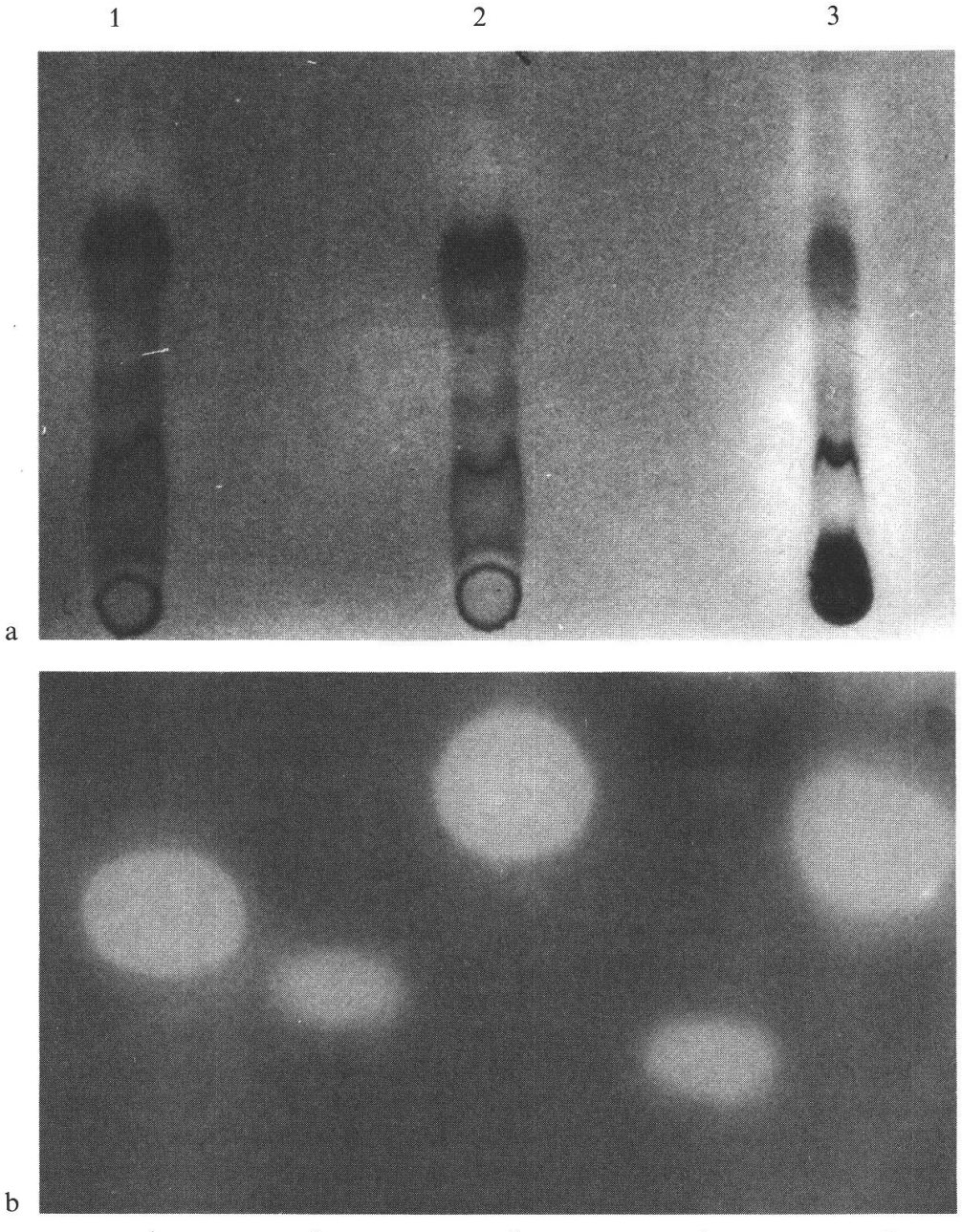

Fig. 2. a From left to right, the tracks show TLC separations of crude extracts from leaves of *Phaseolus vulgaris* cv. Red Mexican, inoculated with streptomycin-killed (lane 1), UV-killed (lane 2) or living cells (lane 3) of a race 1 (avirulent) isolate of *Pseudomonas syringae* pv. *phaseolicola,* respectively. Ethanolic extracts were prepared from leaves 48 h after inoculation, concentrated by rotary evaporation, partitioned against petroleum ether, dried and redissolved in ethyl acetate for TLC. A large inhibition zone extending from the origin in the incompatible combination (lane 3) encompasses several antimicrobial substances. b Inhibition zones caused by purified sesquiterpenoid phytoalexins. Lanes 1–5 are: rishitin, capsidiol, lubimin, 3-hydroxylubimin and debneyol, respectively.

The assay has been used to demonstrate the antibacterial activity, down to 10 μg (the minimum amount tested), of the sesquiterpenoid phytoalexins capsidiol, debneyol, 3-hydroxylubimin, lubimin and rishitin against *P. s.* pv. *phaseolicola* (Fig. 2 b).

Discussion

The assay procedure reported here has great potential in phytopathological research; particularly in view of the controversial status of the antibacterial activity of many compounds tested in different assays by various groups (Table 1). In our assay, coumestrol, kievitone and phaseollin were all shown to be inhibitory to *P. s.* pv. *phaseolicola*. In addition, rishitin was shown to be inhibitory in contrast to a report by Platero Sanz (1981). The assay is rapid, accurate and easy to perform. Absolute age and concentration of the inoculum of the indicator bacteria and the composition of its culture medium are flexible. The need to redissolve the phytoalexins and add them back to growth medium (solid or liquid) to assay their activity is avoided. Presumably, sufficient diffusion of the phytoalexins into the thin agar skin occurs for its antibacterial effects to be apparent.

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