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# ACCUMULATION OF PHYTOALEXINS IN POTATO-CELL SUSPENSION CULTURES

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**Key Word Index**—*Solanum tuberosum*; Solanaceae; potato; cell suspension culture; phytoalexins; rishitin; lubimin; solavetivone.

**Abstract**—Potato-cell suspension cultures (ex Kennebec tuber tissue) accumulate the sesquiterpenoid phytoalexins lubimin, rishitin and solavetivone after inoculation with sporangia of either a compatible or an incompatible race of the fungal pathogen *Phytophthora infestans*. The phytoalexins are present in the cells and the medium. No evidence was obtained for the formation of any metabolites of rishitin.

## INTRODUCTION

Cell suspension cultures of leguminous plants have been used to great advantage for detailed studies of the formation of isoflavonoid phytoalexins (see [1, 2]). As yet, however, tissue culture techniques have not been exploited for studies of the elicitation and biosynthesis of terpenoid phytoalexins, mainly because of the inability of various groups of workers to produce suspension cultures in which it is possilbe to elicit the synthesis of terpenoid phytoalexins. Yamamoto *et al.* [3] detected the noreudesmane rishitin, the major phytoalexin of the potato (*Solanum tuberosum* L.) plant, in potato callus tissue (ex resistant tubers) which had been infected with *Phytophthora infestans* (Mont.) de Bary, the causal agent of late blight, and Érsek and Ziraki [4] found that rishitin and the rearranged eudesmane phytuberin were produced by callus tissue (ex tuber) of the susceptible potato cultivar Desiree (r) when it was inoculated with a mycelial homogenate of *P. infestans*. Henfling [5], however, was unable to show the presence of phytoalexins in either callus tissue or suspension cultures of potato [ex tuber cv Kennebec ($R_1$)] which had been inoculated with either a compatible (Race 1) or an incompatible (Race 4) race of *P. infestans* or an elicitor preparation. He concluded that major- or R-gene resistance was not expressed in these systems and that phytoalexins were not accumulated. Budde and Helgeson [6] found that rishitin plus an isomer, epirishitin, was also produced by tobacco (*Nicotiana tabacum* L.) callus which had been inoculated with *P. parasitica* var *nicotianae*.

Ôba and Uritani [7] reported that sweet potato (*Ipomoea batatas* Lam.) callus (ex root) rapidly produced furanosesquiterpene phytoalexins following a dramatic rise in HMG CoA reductase activity upon transfer to liquid culture and continued to do so if transferred repeatedly into fresh medium. In this system the cells were non-dividing and the phytoalexins were accumulated in the medium. If the callus was not transferred to fresh medium then the HMG CoA reductase activity declined rapidly and phytoalexin accumulation ceased. The accumulation of phytoalexins was dependent upon the presence of yeast extract in the culture medium and a glucan in the extract appeared to be the active component though it was not clear why the extract did not elicit readily detectable levels of phytoalexins in callus tissue grown on the solidified medium. Rather surprisingly, the production of phytoalexins was suppressed if the medium was inoculated with callus tissue and either spores of *Ceratocystis fimbriata* Ell. et Halst, the causal agent of black rot, or $HgCl_2$, an abiotic elicitor of phytoalexin formation.

In this communication it is reported that established potato-cell suspension cultures, like intact tuber tissue, synthesize and accumulate sesquiterpenoid phytoalexins when inoculated with *P. infestans*. This is the first time that terpenoid phytoalexin formation has been elicited in cell suspension culture.

## RESULTS AND DISCUSSION

Potato-cell suspension cultures (ex Kennebec ($R_1$) tuber tissue) were inoculated with either a compatible (complex race) or an incompatible (4) race of *P. infestans*. Twelve hours after inoculation with sporangia from either race of *P. infestans* the potato-cell suspension cultures appeared brown. The cell suspension of the control flask, inoculated with sterile distilled water (SDW), remained white. Twenty one hours after inoculation the cell suspensions were analysed for sesquiterpenoid phytoalexins. By this time the browning response was very pronounced and marginally more intense in the cell suspension inoculated with race 4, the incompatible race.

Tuber tissue discs, the explant tissue of the cultures, also gave a browning reaction with either race of the fungus though the response was slower; the inoculated surface took at least 48 hr to brown to an intensity comparable to that observed in the cell suspension cultures after 21 hr. Tissue darkening, typically browning with the potato-*P. infestans* interaction, is a common response by plants to challenge by micro-organisms or to physical injury [8].

The potato cells and the culture filtrate were analysed separately for phytoalexins. From both of the browned suspensions three compounds were isolated in relatively large quantities (Table 1). These were shown to have the same TLC ($R_f$ and colour reaction) and GC ($RR_t$) properties as authentic samples of the three sesquiterpenoid phytoalexins solavetivone ($R_f$ 0.44, buff, $RR_t$ (methyl arachidate) 0.56), rishitin (0.21 blue, 0.72) and lubimin (0.28, turquoise, 1.20). In addition GC/CI($NH_3$)MS gave major ions (rishitin, $m/z$ 240, $[M + NH_4]^+$; lubimin 254, $[M + NH_4]^+$; solavetivone 236, $[M + NH_4]^+$) consistent with the known MWs of these phytoalexins and GC/EIMS gave the expected fragmentation ions. No phytoalexins were detected in the white cell suspension from the control flask.

Unlike the chloroform-soluble lipid of the whole cells, the ether extract of the culture medium required no chromatographic purification prior to GC analysis. The large proportion of each phytoalexin in the culture medium (Table 1) suggests that the phytoalexins diffused into the medium from the cells in which they were synthesized, as occurs with the furanosesquiterpenoid phytoalexins synthesized by non-dividing cells of sweet potato callus in response to suspension in fresh culture medium [7]. These observations are consistent with the view that in host tissues infected with an incompatible pathogen phytoalexins are synthesized in healthy cell layers and then move out and accumulate in the adjacent necrotic tissue [9, 10].

In infected discs of explant tissue only rishitin and lubimin were detected, the former accumulating as the major phytoalexin (Fig. 1.) This pattern was clearly reversed with the cell suspensions, in which in addition solavetivone was present (Table 1). The total amounts of phytoalexins accumulated per unit weight of harvested cells (i.e. μg phytoalexins (cells plus medium)/g fr. wt) from the suspension cultures inoculated with *P. infestans* were some two times greater than the highest amounts accumulated in the infected tissues of the explant tissue. However, despite this increase in phytoalexin accumu-

Table 1. Phytoalexin content of potato-cell suspension cultures inoculated with either an incompatible (Race 4) or a compatible (complex) race of *P. infestans*

| Fungus | Wt. of cells (g)* | Phytoalexin (μg)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | Lubimin | | Rishitin | | Solavetivone | |
| | | Cells | Medium | Cells | Medium | Cells | Medium |
| Complex race | 24 | 225 | 622 | 19 | 44 | 95 | 174 |
| Race 4 | 31 | 197 | 685 | 50 | 163 | 73 | 141 |

* From 100 ml of suspension culture inoculated with 1 ml of sporangial suspension.

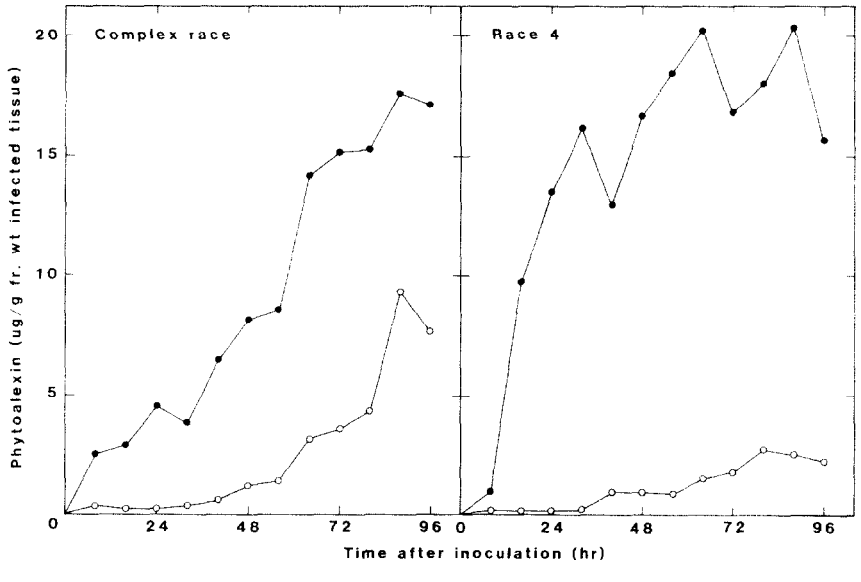


Fig. 1. Time-courses for the accumulation of phytoalexins in aged discs of explant tuber tissue inoculated with *P. infestans*. ●—●, rishitin; ○—○, lubimin. 2 ± 0.2 g infected tissue (first mm) was taken for each analysis. Microscopic examination of stained tuber discs revealed that the complex race of the fungus grew through the 5 mm tuber discs whereas race 4 did not penetrate beyond the first mm.

lation, the amounts of rishitin accumulated in the suspension cultures were lower than those in the infected explant tissue.

In view of the findings that endogenously supplied samples of lubimin and solavetivone are efficiently converted into rishitin by potato tuber tissue [11], the marked accumulation of these two presumptive precursors in potato-cell suspension cultures inoculated with *P. infestans* may represent yet another example of the build-up of the biosynthetic intermediates of a secondary metabolite in cell suspension culture. Commensurate with *de novo* synthesis of lubimin, rishitin and solavetivone, the three phytoalexins are readily labelled with $^{14}$C-mevalonate and there is evidence, as in the potato tuber discs [P. A. Brindle, unpublished work], that sterol synthesis is strongly suppressed during phytoalexin synthesis.

The failure of Henfling [5] to obtain phytoalexin formation in potato tissue cultures obtained from the same explant and treated with similar races of *P. infestans* as used in the present investigation is presumably a reflection of the well-known variability associated with the formation of secondary metabolites by cell cultures or may simply be due to differences in experimental techniques.

The fact that the Kennebec cell suspensions did not accumulate 13-hydroxyrishitin is perplexing in view of the work of Ward *et al.* [12] who demonstrated that healthy cell suspensions of the same potato cultivar metabolised exogenously supplied rishitin and lubimin to 13-hydroxyrishitin and unidentifiable lubimin metabolites, respectively. This apparent variance may be reconciled at a later date when further studies have been made with the cell culture system described here.

## EXPERIMENTAL

*Biological methods. Phytophthora infestans*, race 4 (Hull University culture collection) and a complex race known to be able to match the $R_1$ major gene (a generous gift from Dr. D. D. Clarke, Glasgow University), was maintained on bean agar at 18° ± 3° and subcultured monthly [13]. The agar was supplemented with cholesterol and sitosterol at 50 μg/ml to promote sporangia formation [14]. Sporangial suspensions were obtained by flooding 9–12 day old cultures with sterile distilled water (SDW).

Tubers of *Solanum tuberosum* cv Kennebec ($R_1$) were obtained from a commercial grower in Aberdeen, Scotland. They were lifted in September 1981 and stored at 4°, in darkness, until used. Discs (15 mm dia × 5 mm) of tuber tissue were prepared from 8 month old tubers that had been equilibrated to room temp. and surface sterilized with 15% Everchlor for 15 min. Each disc was rinsed with SDW (× 3) and aged for 24 hr on 1% water–agar at 18° ± 3°, in darkness, before inoculation of the upper surface with 0.05 ml of sporangial suspension ($1.2 \times 10^5$ sporangia/ml) or SDW.

Callus was initiated from 8-month-old tubers. Under aseptic conditions, discs (5 mm dia × 0.5 mm) were prepared from a surface sterilized tuber as described earlier. The explants were rinsed with SDW (× 3) and cultured at 27°, in darkness, on a modified 0.8% agar medium (RM-1964) [9]; the developing calli were transplanted every 4 weeks. To initiate cell suspensions, clumps of callus were transferred to 50 ml of liquid medium in a 250 ml conical flask and shaken, in darkness, at 150 rpm in a Gallenkamp Orbital incubator at 27°. Every three weeks 10 ml of the cell suspension was transferred to 90 ml of fresh medium. Each experimental suspension (100 ml vol) was in the 21st day of its 5th subculture and was inoculated with 1 ml of sporangial suspension ($8 \times 10^5$ sporangia/ml) or SDW.

*Analytical methods.* The first millimetre from the top surfaces of infected tuber discs and the water-washed whole cells from the filtered cell suspensions were extracted with $CHCl_3$–MeOH (2:1, 5 ml/g fr. wt). The $CHCl_3$-soluble lipids were subjected to TLC on silica gel G (0.75 mm) developed with cyclohexane–EtOAc (1:1, phytoalexins located with vanillin–$H_2SO_4$ [7]). Compounds corresponding to authentic phytoalexins were eluted with $Me_2CO$ for analysis by FID/GLC (GCD chromatograph, Pye Unicam): glass column (2.1 m × 2 mm) packed with 3% OV225 on Gas Chrom. Q (100–200 mesh); $N_2$, 35 ml/min; inj., 225°; det., 300°; col., 180°. Phytoalexins were quantified on the basis of their peak areas relative to that of the internal standard methyl-arachidate using an integrator.

The filtrate and washings from the cell suspensions were combined and partitioned (× 3) against an equal vol. of $Et_2O$. The combined $Et_2O$ extracts were taken to dryness, redissolved in cyclohexane and analysed by GC. GC/MS data were obtained with a Finnegan Quadrapole Mass Spectrometer by Dr. F. Cottee, Shell Research Ltd., Sittingbourne. The GC conditions were similar to those described already.

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