Cytochalasins A and B from Strains of *Phoma exigua* var. *exigua* and Formation of Cytochalasin B in Potato Gangrene

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INTRODUCTION

On the basis of a correlation between the severity of potato late blight (caused by *Phytophthora infestans*) and incidence of anencephaly and spina bifida in humans, Renwick (1972, 1973) proposed a hypothesis that 95 % of the occurrences of these birth defects could be prevented by avoidance of the potato during early pregnancy. This hypothesis was not restrictive to direct involvement of late blight. Shepard (1973) made the suggestion that cytochalasin B, which has been shown to produce neural tube closure defects in explanted chick embryos (Linville & Shepard, 1972), might be the teratogenic agent. Cytochalasins A and B, which are compounds possessing unusual activity toward cultured cells (Carter, 1972), are known to be produced by *Helminthosporium dematioideum* and a *Phoma* sp. (Aldridge, Armstrong, Speake & Turner, 1967; Rothweiler & Tamm, 1970). It therefore appeared desirable to examine strains of potato pathogens belonging to the same genera, in particular *Helminthosporium atrovirens* (*H. solani*) and *Phoma exigua*, for cytochalasins A and B and related compounds. *Helminthosporium atrovirens* causes 'silver scurf' (Connors, 1967) while two varieties of *P. exigua*, *P. exigua* var. *exigua* and *P. exigua* var. *foveata*, are responsible for potato gangrene (Logan & O'Neill, 1970).

METHODS

Organisms. Six isolates of *H. solani* were obtained from silver scurf lesions that developed during incubation of market potatoes at 12 °C under humid conditions. One other isolate of this species was from J. Santerre, Agriculture Canada, Ste. Foy, Quebec. One strain of *P. exigua* var. *exigua* (HPB070673-1) was isolated in this laboratory from a diseased potato by incubation of the lesion on Czapek-Dox agar containing 200 p.p.m. streptomycin. Other strains of *P. exigua* were from C. Logan, Ministry of Agriculture, Belfast, Northern Ireland (two var. *exigua*, six var. *foveata*), R. L. Griffith, Rothamsted Experimental Station, Harpenden, Hertfordshire (three var. *exigua*, three var. *foveata*), and from C. H. Lawrence (five var. *exigua*) and W. A. Hodgson (one var. *exigua*, HPB220873), Agriculture Canada, Fredericton, New Brunswick. In addition, one isolate of *Colletotrichum coccodes* ('black-dot' organism) from J. Santerre, three isolates of *Rhizoctonia solani* ('black scurf' organism) from R. L. Griffith, three strains of *Alternaria alternata*, and one strain of *Alternaria tenuissima* isolated from potato were examined.

Maintenance and culture. Single spore or single hyphal tip isolates from fungal cultures were maintained on potato carrot agar or malt extract agar. To screen for cytochalasin production, they were grown on 5 ml liquid medium (Rothweiler & Tamm, 1970) in 30 ml screw-cap vials maintained in a sloping position for 7 days at 25 °C. Cultures were shaken with 10 ml chloroform, heated at 65 °C for 5 to 10 min, and allowed to stand with the solvent,

usually for about 15 min at room temperature. Experiments with *H. dematioideum* (CMI-74812) and Phoma strain s298 (from E. Härri, Sandoz A.G., Basel, Switzerland) verified the efficacy of these procedures for production and extraction of cytochalasins A and B. For large scale culture, two strains of *P. exigua* var. *exigua* (HPB220873 and HPB070673-1) were grown for 9 days at room temperature in 2.8 l Fernbach flasks containing 250 ml medium per flask. Culture medium was extracted directly with chloroform and mycelium was boiled in chloroform, then soaked in the solvent for 3 h.

Thin-layer chromatography. Extracts were concentrated to about 0.5 ml for examination by t.l.c. on 0.25 mm precoated layers of silica gel DB (Camag). For use as t.l.c. standards, cytochalasins A and B were purchased from I.C.I. Ltd (a sample of the latter was also received from C. Tamm); cytochalasins C and D (Aldridge & Turner, 1969; Minato & Matsumoto, 1970) were gifts from H. Minato; and cytochalasin E (Aldridge, Burrows & Turner, 1972; Büchi *et al.* 1973) was from R. C. Shank. Standard solutions of cytochalasins A, B, C, D (100 µg each/ml chloroform) and E (200 µg/ml) were spotted, together with 5 µl extract. Toluene-ethylacetate-90% formic acid (5:4:1, by vol; TEF) and, for confirmation, chloroform-acetone (9:1, v/v; CA) and chloroform-methanol (93:7, v/v) were used as developing solvent systems. Chromatograms were sprayed with 65% (v/v) sulphuric acid then heated at about 100 °C for 3 to 4 min to allow observation of cytochalasins A and B as blue fluorescent spots under reflected and transmitted longwave u.v. light; longer heating at 120 °C produced a grey to yellow fluorescence. Cytochalasins C and D formed yellow fluorescent spots while cytochalasin E gave a reddish-brown fluorescence and a grey spot in visible light. Approximate estimations of cytochalasin B were made after t.l.c. of rediluted extracts by visual comparison of fluorescence intensities with those of the standard. Cytochalasins A and B were isolated from concentrated medium extracts of large scale cultures by preparative t.l.c. on 0.5 mm layers of silicAR 7 GF (Mallinckrodt) by using solvent system CA for development, and crystallized from benzene-hexane and benzene, respectively.

Potato cultures. The cut surfaces of market potatoes, from which a 1 cm layer had been removed, were inoculated with each of two cultures of *P. exigua* var. *exigua* obtained from single hyphal tips of strain HPB220873 and incubated at 8 °C on dry towels for three weeks, then on moistened towels for a further three weeks. Rot (73 and 94 g) was excised and blended with 200 or 250 ml methanol-water (55:45, v/v). The mixture was centrifuged at 700 g for 5 min and a measured volume of extract shaken with an equal volume of chloroform. Filtration of the chloroform extract through phase-separating filter paper (Whatman PS 1) was necessary to clear emulsions, then a portion was evaporated to dryness and analysed by two-dimensional t.l.c. using the solvent systems CA and TEF for first and second developments, respectively. Presumptive cytochalasin B was purified by repeated preparative t.l.c. and confirmed by mass spectroscopic comparison with the standard at a probe temperature of 165 to 170 °C (Perkin Elmer RMS-4 instrument operated at 80 eV).

RESULTS AND DISCUSSION

Of the species examined, only *P. exigua* var. *exigua* produced any of the cytochalasins looked for. Our findings support the observation of Bousquet & Barbier (1972) that this species produces cytochalasin B. Cytochalasins A and B were found in all 12 strains of this variety, isolated from potatoes in Canada, Northern Ireland and England. Amounts of cytochalasin B produced in the small vial cultures ranged from 40 to 200 µg/ml medium and cytochalasin A was consistently present in small amounts (< 20 µg/ml), although up to 100 µg/ml was found in the medium from the reference fungus *H. dematioideum*. No cyto-

challasins C, D or E were detected in any isolate. Typical R_F values for cytochalasins A, B, C, D and E were 0.60, 0.50, 0.38, 0.30 and 0.42, respectively, in solvent system TEF, and 0.80, 0.62, 0.26, 0.23 and 0.23 in solvent system CA. Separation of cytochalasins A and B was better in the latter system and also in chloroform-methanol (93:7), but TEF was best for separation of all five cytochalasins. Minimum detectable amounts on the t.l.c. plate were of the order of 0.1 μ g. Crystalline cytochalasin A (m.p. 184 to 186 °C and 188 to 189 °C) and cytochalasin B (m.p. 220 to 221 °C, 216 to 217 °C) were isolated from large scale cultures of two of the strains of *P. exigua* var. *exigua*. Their identities were confirmed by mixed m.p., and infrared, quantitative u.v., and mass spectroscopic comparisons with authentic cytochalasins A and B. Thin-layer chromatographic analysis indicated that approximately equal amounts of cytochalasin B were present in the culture medium and mycelium; total estimated yields were 70 mg/l medium.

It is of particular interest that none of the nine strains of *P. exigua* var. *foveata* examined formed cytochalasins A or B. This variety has been subdivided on the basis of whether a substance named Antibiotic 'E' is produced or not (Logan & O'Neill, 1970) and we screened strains of both types. *Phoma exigua* var. *foveata* has been reported to be the main cause of potato gangrene infections in Northern Ireland (Logan & O'Neill, 1970). In Canada, on the other hand, the pathogen causing phoma rot of potato has usually been termed *Phoma tuberosa* (synonymous with *P. exigua* var. *exigua*) (Boerema & Höweler, 1967; Conners, 1967).

Phoma exigua var. *exigua* was shown to form cytochalasin B on unsterilized potato. Approximate concentrations found were 1 and 5 μ g/g potato in the excised rot caused by the two cultures of strain HPB220873. The extraction procedure used had previously been found to give 100 % recovery of cytochalasin B added at a concentration of 10 μ g/g to sound potato. Two dimensional t.l.c. was necessary to analyse cytochalasin B in the rot at the low concentrations found. Further work will be necessary to determine optimum conditions for production of cytochalasin B in the potato. Poswillo *et al.* (1973) reported the presence of weak cytochalasin activity (binucleation in cultured L cells) in extracts from potato concentrates that produced foetal malformations in the marmoset; these potatoes were naturally infected with *Phytophthora infestans* but other fungi might have been present.

We have demonstrated a direct association between cytochalasin B and one variety of potato gangrene. If further research indicates cytochalasin B as a teratogen *in vivo*, then a search for naturally occurring cytochalasin B would be warranted. Recent studies on cytochalasin B fed to pregnant rats in daily doses of 1 mg/kg body weight have, however, yielded negative results (Ruddick, Harwig & Scott, 1974).

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