Induction of Common Scab Symptoms in Aseptically Cultured Potato Tubers by the Vivotoxin, Thaxtomin

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ABSTRACT

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Inoculation of aseptically cultured minitubers with cell-free extracts from scab lesions of field-grown and cultured tubers infected with *Streptomyces scabies* reproduced symptoms typical of the common scab disease. Isolation and fractionation of the active components in tissue extracts by a combination of normal and reversed phase thin-layer chromatography yielded two active compounds. These have been designated thaxtomin A and thaxtomin B and characterized as unique 4-nitroindol-3-yl containing 2,5-dioxopiperazines. These phytotoxic principles satisfy all of the basic criteria of vivotoxins.

Despite attempts made over the past 60 yr to elucidate the host-parasite interaction in the common scab disease of potatoes, we still lack a clear-cut picture of the mechanism by which the pathogen, *Streptomyces scabies*, is able to induce the development of lesions in the potato tuber.

Among the first to offer an explanation of the underlying events in scab development was Fellows (3), who noted the darkening of the tuber cell walls in advance of colonization by the pathogen and concluded that this symptom was a response by the tuber

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to the action of a toxin or enzyme elaborated by the scab organism. This conclusion received some indirect support from Jones (4), who reported on the localization and confinement of the pathogen to the periphery of the developing lesions and on the concomitant absence of hyphal filaments or their remnants in the cells adjacent to the lesions.

So far, however, the only direct evidence for the toxin concept has come from a single source. Shoemaker (8) reported that he was able to induce scab lesions by transferring small agar blocks taken from the proximity of nonsporulating colonies of *S. scabies* to the surface of tubers maintained under sterile conditions. Subsequent plating of samples from the typical scab lesions that developed failed to yield actinomycetous colonies in culture medium, and taken together these observations were interpreted as evidence for the induction of common scab by "a diffusible metabolic substance" originating in the pathogen. But this conclusion was questioned when it was subsequently revealed that samples taken from the 'sterile' tubers showed the presence of one bacterial and three fungal contaminants.

The present investigation was undertaken as part of an attempt to provide further evidence for the role of toxins in the etiology of the scab disease and is based on the use of aseptically cultured minitubers as a reliable means for assaying scab-inducing activity in a cell-free system. We report here on the methods and procedures that were employed in the isolation, purification, and characterization of two toxic components obtained from scabby tissue in both field-grown and aseptically cultured minitubers.

MATERIALS AND METHODS

Aseptic tubers of *Solanum tuberosum* L. 'Green Mountain' were produced and maintained in vitro by implanting sterile sections from dark-grown sprouts in White's amended medium (1) containing 8% sucrose (7). Minitubers were harvested and tested for their reaction to the organism and to scab-inducing compounds at all stages of their development.

Different strains of *S. scabies* were isolated from diseased tubers in the field (6). All isolates were maintained and routinely subcultured on Czapek's agar medium. Pathogenicity of the strains was verified either by inoculating disease-free, surface-sterilized tubers in pots under greenhouse conditions, or by inoculating minitubers under aseptic conditions.

Scabby tissue from infected tubers was excised and homogenized in acetone to produce a fine slurry. This was filtered under vacuum on Whatman No. 1 paper, and the filter cake was extracted twice with equal quantities of acetone. The filtrates were combined and the acetone was evaporated in vacuo at room temperature. The remaining aqueous suspension was extracted twice with equal volumes of chloroform. The chloroform was removed by evaporation at reduced pressure at room temperature, and the residue was taken up in acetone and filtered on Whatman No. 1 paper in order to remove coextracted waxes. The acetone was evaporated at reduced pressure and the residue was fractionated by thin-layer chromatography on 0.25 mm Silica gel 60 with chloroform/methanol (9:1), followed by a further separation on 0.2-mm RP-C_{18} with acetone/water (3:2).

Crude extracts, partially fractionated material, and purified components were routinely assayed for scab-inducing activity by absorbing 10–20-μl droplets of the test sample (suspended in water and filtered through a 0.22-μm filter) on 4-mm antibiotic blank paper disks and appressing these directly onto the surfaces of sterile minitubers. Lesions usually appeared within 24 hr. Test fractions also were routinely checked for the presence of contaminating microorganisms by plating them on Czapek's, potato-dextrose, or nutrient agar medium.

In situ assays of the scab-inducing principle were performed according to the following protocol. A small piece of sterile moistened dialysis tubing was placed on the scab lesion and overlayed with a sterile healthy minituber. Diffusion was allowed to take place across the interface and the extent of reaction determined by visual inspection after 24–48 hr. The dialysis tubing used in the transfer was checked for microbial contamination as described above.

Quantities of the active material sufficient for chemical characterization and structural analysis were obtained from field-grown tubers. The tubers were washed, peeled, and sliced; and these were surface-sterilized, transferred to sterile petri plates, and inoculated with a suspension of *S. scabies*. After 7 days, when the slices were thoroughly infected, the scab-inducing material was extracted and purified by TLC according to procedures described above.

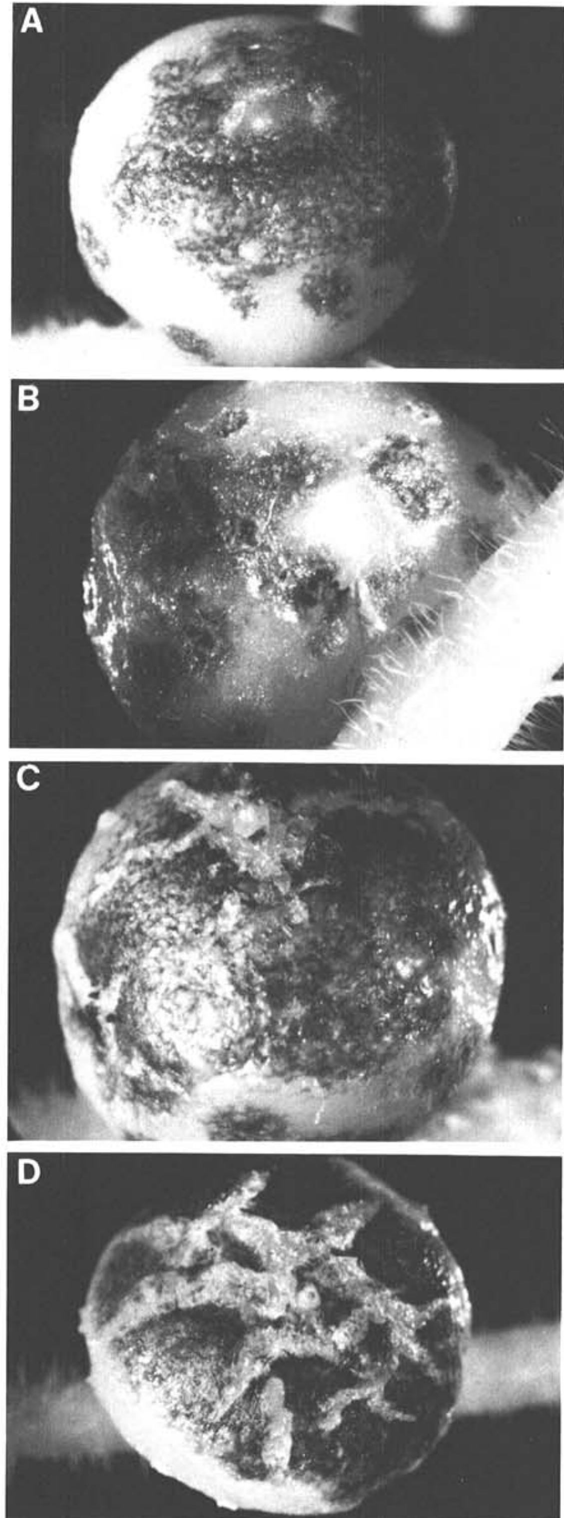

Fig. 1. Reaction of cultured potato tubers to inoculation, **A and C**, with the scab-inducing principle and, **B and D**, with the common scab organism.

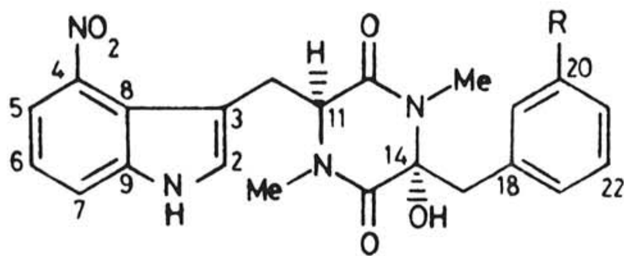

Fig. 2. Structural formulae for (A) thaxtomin A and (B) thaxtomin B.

RESULTS

A comparison of the symptoms elicited in aseptically cultured minitubers by the scab organism with those of the scab-inducing material can be made from the composite photograph in Figure 1. The reactions shown in Figure 1A and C are typical of early (24 hr) and late (1 wk old) symptoms, respectively, elicited by the scab-inducing material; those shown in Figure 1B and D are those obtained with the actual scab organism after 1 and 2 wk, respectively.

Close examination of minitubers inoculated by both methods made it clear that the entire range of visible symptoms typical of the common scab disease was present in both cases. Cell proliferation and expansion and the subsequent eruption and browning of tissue surrounding the inoculation zone, as well as the eventual cracking of the tuber surface, were indistinguishable in both interactions, as revealed particularly by comparison of Figure 1C and 1D.

Thin-layer chromatography on silica gel plates of material isolated from scab lesions gave two active fractions: A major spot at R_f 0.27 and a minor one at R_f 0.41. A second separation of these two fractions on RP-C_{18} yielded zones of scab-inducing activity at R_f values of 0.65 (minor) and 0.77 (major), which correlated with yellow colored components.

After rechromatography on silica gel these components were induced to crystallize from acetone/methanol. The major product, thaxtomin A (Fig. 2A), crystallized as light orange rosettes, mp 230 C (decomp.); M^+ 438.1708; λ_{max} (EtOH) 398 (ϵ 4,050), 343 (ϵ 3,220), 279 (ϵ 5,830), 249 (ϵ 15,070), and 220 (ϵ 27,700) nm: μ_{max} (Nujol) 3,200–3,380, 1,650, 1,600, and 1,510 cm^{-1} and was characterized by spectral methods and partial synthesis (5) as the 2,5-dioxopiperazine shown in Figure 2.

The minor product, thaxtomin B (Fig. 2B) crystallized as light orange needles mp 238 C (decomp.); M^+ 422.1753 and was assigned a structure based on a comparison of its spectral properties with those of thaxtomin A. Further details on the purification, structural elucidation, and partial synthesis of these compounds have been published elsewhere (5).

Yields of purified vivotoxin from infected tubers averaged 7.2 $\mu g/g$ of tissue for thaxtomin A and 0.4 $\mu g/g$ for thaxtomin B. These compounds showed equivalent scab-induction at 10^{-5} and 10^{-6} M.

DISCUSSION

In formulating the criteria by which a given phytotoxic agent or substance could be classified as a vivotoxin, Dimond and Waggoner (2) proposed that the burden of proof for complicity in the disease reaction should rest primarily on whether the substance in question could be reproducibly separated from the diseased plant and then used to reproduce at least a portion of the disease in healthy plants. Clearly, the scab-inducing compounds, thaxtomin A and thaxtomin B, satisfy both prerequisites. Moreover, it is also evident that these compounds are highly specific for this particular interaction since none of our many attempts to induce their production in healthy tubers by physical or chemical injury or by inoculation with two nonpathogenic *Streptomyces* species: *S. echinatus* (ATCC 19748) and *S. triostinicus* (ATCC 21043) has been successful. We should stress, too, that a variety of different culture media failed to show any evidence of thaxtomin production when inoculated with pathogenic strains of the scab organism.

This latter observation contradicts the findings of Shoemaker (8), and we can only conclude that the contaminants that he isolated from inoculated tubers did in some way interfere with his attempts to isolate actinomycetous colonies from the scab lesions.

Finally, we should point out that the biogenesis of the thaxtomins in this host-parasite interaction appears in each case to involve a combination and *N*-methylation of two modified amino acids, i.e., α-hydroxy-*m*-tyrosine plus 4-nitrotryptophan for thaxtomin A and α-hydroxyphenylalanine plus 4-nitrotryptophan for thaxtomin B. The presence of the nitro group in the tryptophan indole ring is unusual and means that the thaxtomins represent a new variation on phytotoxic compounds.

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