Isolation and Identification of Xanthotoxin (8-Methoxypsoralen) and Bergapten (5-Methoxypsoralen) from Celery Infected with Sclerotinia sclerotiorum

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Methods for the production, isolation, and identification of xanthotoxin and bergapten from celery diseased by Sclerotinia sclerotiorum (Libert) de Bary were investigated. The only conditions under which this mold was capable of producing xanthotoxin and bergapten occurred when the mold was actively growing on fresh (metabolizing) celery. Neither compound was found in uninfected celery, in the mold growing on nutrient media, on nutrient media fortified with 30% filter-sterilized celery juice, or on nonmetabolizing celery. Maximal xanthotoxin production of 320 μg per g of dry rotted celery occurred at 20°C, although mycelial growth increased until 30°C. Neither xanthotoxin nor bergapten was found when the mold grew on 11 agricultural commodities other than celery.

Sclerotinia sclerotiorum attacks many kinds of fruits and vegetables in the field as well as in storage, in transit, and in the market (2, 8). Sclerotinia-diseased celery (pink rot) was found to cause a blistering cutaneous disease on the skin of people working in celery fields (1, 6). The skin-sensitizing phototoxins xanthotoxin (8-methoxypsoralen), 4,5',8-trimethylpsoralen and bergapten (5-methoxypsoralen) have been reported in diseased celery (3, 5, 6, 9).

The environment necessary for the production of these furanocoumarin toxins was investigated. A convenient method of producing these compounds for laboratory experimentation is described. An efficient system for extracting these compounds and a thin-layer chromatographic (TLC) system suitable for their identification and quantitation were developed. By using these methods, the production of phototoxins on pink-rot celery was determined at various temperatures. Also, the study was extended to determine whether the infection of S. sclerotiorum on other vegetables could cause a phototoxicological hazard.

MATERIALS AND METHODS

Organisms. S. sclerotiorum (ATCC 10939), originally isolated from the lima bean, was used throughout this study. Peptone-dextrose-yeast extract (PDYE) broth (11) containing 2% peptone, 6% dextrose, and 2% yeast extract in distilled water was used to propagate the mold. Celery was procured from local grocery stores. Only healthy stalks of approximately the same size with some root remaining were selected. Upon arrival at the laboratory, the celery plant was dipped into 10% NaOCl solution for 2 min and then rinsed for 2 min with distilled water before inoculation. Other vegetables were selected and treated in a similar manner.

Standards. Xanthotoxin was purchased from Mann Research Laboratories, Inc., Orangeburg, N.Y., and 4,5',8-trimethylpsoralen was provided through the kindness of Paul B. Elder Co., Bryan, Ohio. Reagent grade acetone was used for preparing standard solutions.

Quantitative inoculation. A large amount of fungus mycelium was obtained by growing S. sclerotiorum in PDYE broth for 2 weeks. The mycelial mat was blended aseptically with a small amount of the original broth to a concentrated, homogenized hyphal solution. Inoculation was made by pipetting equal amounts of hyphal solution onto the surface of the test plants or into PDYE broth. The quantity of mycelium pipetted was determined from the dry weight of mycelia found by pipetting the same amount of hyphal solution into a test tube, centrifuging, and weighing after drying at 105°C for 2 hours.

Production of phototoxins: (i) S. sclerotiorum in PDYE broth. Mycelium of S. sclerotiorum was transferred into each of three 500-ml Erlenmeyer flasks containing 150 ml of PDYE broth, and the
inoculated flasks were incubated at 25 C for 10 days.

(ii) S. sclerotiorum in PDYE broth with 30% celery juice. Mycelium was transferred to a celery juice broth prepared by blending one part of fresh celery with two parts of distilled water (w/w) and filtering through cheese-cloth and Whatman no. 1 filter paper. The juice was sterilized by using a Nalgene filter unit with a 0.45-μm membrane. Sterile celery juice was added to autoclaved PDYE broth to obtain a final 30% celery content.

(iii) S. sclerotiorum on steamed celery pieces. A 100-g amount of celery stalks was autoclaved at 110 C for 15 min. Mycelium grown for 2 weeks on PDYE broth was transferred to the steamed celery stalks. Cultivation was at 25 C until mold growth appeared.

(iv) S. sclerotiorum on living celery. The treated celery was placed upright in a water trough inside a sealed, plastic cultivating chamber. The water in the trough covered the root base of the celery. To give a 1% concentration, three drops of a liquid fertilizer (10:10:10) and NaOCl were added to the water. Wounds, made into a shape convenient for holding the measured amount of mycelial solution, were made with a sterile knife in two different areas on each celery stalk. The culturing chamber was closed, sealed with Scotch 202 masking tape (3M Co., Minneapolis, Minn.), and incubated at 25 C for 1 week to obtain infection. Uninoculated celery was incubated simultaneously as a control.

Extraction method. One-half volume of test solvent was added to liquid samples and, after shaking for 5 min on a gyratory shaker, the mixture was poured into a separatory funnel and allowed to separate into two phases. Then the solvent phase was collected. The procedure was repeated twice to ensure collection of all toxin. Solid samples were ground and lyophilized. Batches of 4 g of the sample, dry-blended to a homogeneous powder, were shaken with 50 ml of test solvent in the same manner as the liquid sample except that the extraction solvent was collected by filtration. Shaking and filtration were repeated three times. The extracting solution from either liquid or solid samples was concentrated to 5 ml by evaporation at 40 C under reduced pressure on a flash evaporator. Samples of 5 μl from this concentrated solvent were spotted on Silica Gel G TLC plates and developed in benzene-ether (2.5:1).

Identification and quantitation of phototoxins. Several different TLC absorbent and solvent systems were compared to obtain the best resolution. The developed plate was observed under long-wave ultraviolet light and the separated spots were identified routinely by comparison of λ<sub>max</sub> and fluorescence color with standards. Identification was confirmed by comparison of the ultraviolet spectra of the eluted TLC spots with reference spectra (9). The identity of the compounds was further established by the production of a photosensitized erythermal reaction (6) on rabbit skin by using an ethyl acetate solution of the suspected phototoxin isolation by preparative TLC.

The smallest amount of xanthotoxin per spot easily observable under ultraviolet light was found to be 0.5 μg. The compounds separated on 0.25-mm Silica Gel G TLC plates were quantitated with a fluorodensitometer (Photovolt Corp., New York, N.Y.). A 525 ± 20-nm narrow-pass filter was used on the detector. For each plate, a curve of intensity (peak area) versus concentration was made from standards, and the concentration of the sample spot was determined from the standard curve.

RESULTS AND DISCUSSION

Extraction efficiencies of eight solvents were compared (Fig. 1) to select the best solvent for quantitatively extracting small samples for separation and quantitation of furocoumarin phototoxins by TLC. Of the solvents tried, ethyl acetate, which was nearly five times more efficient than petroleum ether, was the strongest extracting solvent for xanthotoxin. The results show that ethyl acetate was also a much more efficient solvent for extracting bergapten from diseased celery than were other common solvents. Although ethyl acetate was the most efficient extractant for xanthotoxin and for bergapten, many compounds other than the one sought are also extracted by ethyl acetate (Fig. 2). Separation methods other than crystallization are required if ethyl acetate is used. Probably for this reason, petroleum ether often was chosen in the past to assure higher purity during crystallization.

New techniques such as paper, column, and thin-layer chromatography have been used in separating psoralens from higher plants as modern analytical techniques have become available. However, most of the procedures require that the components be purified before separation. If the preparations are not purified beforehand, most procedures merely separate psoralens from other compounds without resolving psoralen compounds from each other. To obtain a high-resolution, one-step quantitative assay, an improved thin-layer chromatography system was developed. Silica Gel G, slurried in 0.3 M sodium acetate, gave the best separation of the adsorbents tested. A number of developing solvents were compared (Table 1). Chloroform (12) separated xanthotoxin and bergapten well, but each overlapped other compounds and the migration of the solvent was very slow. Both benzene-ethyl acetate (9:1) (4) and benzene-ether (1:1) (3) separated psoralens from other compounds, but the latter gave better resolution between the two psoralens. Petroleum ether-chloroform (4:1) (7) and toluene-ethyl formate-formic acid (5:4:1) (10) were not satisfactory for a single-step identification of psoralens from pink-rot celery.

Different solvent ratios of the benzene-ether system were studied to obtain the best separation. A 2.5:1 ratio of benzene-ether (Table 1)
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Fig. 1. Efficiency of solvents relative to petroleum ether for removing xanthotoxin and bergapten produced in celery diseased by Sclerotinia sclerotiorum.

Fig. 2. Fluorodensitometric tracing of a thin-layer chromatogram of petroleum ether (broken line) and ethyl acetate (continuous line) extracts of Sclerotinia-diseased celery.

gave the best separation of a direct ethyl acetate extract of psoralen compounds from S. sclerotiorum-diseased celery.

A suitable system for the identification of furanocoumarin compounds from celery diseased by S. sclerotiorum involved extraction of the sample material with ethyl acetate followed by thin-layer chromatography on plates prepared from Silica Gel G slurried in a 0.3 M sodium acetate solution and developed in ben-
TABLE 1. Developing systems for identification of psoralens from infected celery*

| Solvents              | Separation from other compounds | Resolution between psoralens | Developing time (min) |
|-----------------------|---------------------------------|------------------------------|-----------------------|
| Petroleum ether-chloroform (4:1) | None                            | Poor                         | 55                    |
| Toluene-ethyl formic acid (5:4:1) | Very poor                       | Poor                         | 45                    |
| Benzene-ethyl acetate (9:1) | Poor                            | Good                         | 90                    |
| Benzene-ether (1:6)     | Poor                            | Fair                         | 45                    |
| Benzene-ether (1:1:1)   | Fair                            | Good                         | 45                    |
| Benzene-ether (2.5:1)   | Good                            | Good                         | 50                    |
| Benzene-ether (8:1)     | Very poor                        | Fair                         | 66                    |

* Silica Gel G slurred with 0.3 M sodium acetate. Evaluations are based on the extent of peak overlap observed on the thin-layer chromatography fluorodensitometer tracings of the developed plates.

zene-ether (2.5:1) after activation for 30 min at 105 C. Quantitation of the psoralens was readily accomplished with speed, convenience, and accuracy by comparing the fluorescence of the TLC psoralen spots with fluorodensitometry. The intensity of the fluorescence of TLC spots was measured without interference from contaminants in the solvents. The accuracy of fluorodensitometry depended, however, on the uniformity of the thickness of the TLC adsorbent layer, the resolution of the spots, and the concentration of the compound in the spot to be measured. In this study, a four-point standard calibration curve was prepared for each individual TLC plate. This system was found to be adaptable to most fruit or vegetable samples.

In the literature (3, 6, 9), it is usually mentioned only that phototoxic furanocoumarins were isolated from S. sclerotiorum-diseased celery. It was hoped that a method could be developed to cultivate the mold in a simple medium to obtain toxin for study more conveniently. To this end, various culture methods were analyzed for toxin production using the method developed in this study.

The uninfected celery, the mold growing in PDYE broth, the mold growing in PDYE broth plus 30% filter-sterilized celery juice, and the mold growing on steamed celery stalks did not produce xanthotoxin, bergapten, or 4,5',8-trimethylpsoralen. The only growing conditions under which S. sclerotiorum was capable of producing xanthotoxin and bergapten occurred when the fungus was actively growing on fresh celery. Three strains, ATTC 10939, ATTC 18015, and the strain of Floss et al. (3), all failed to produce xanthotoxin or bergapten on nutrient media. In contrast to the results reported by Scheel et al. (9), no 4,5',8-trimethylpsoralen was produced by this strain of S. sclerotiorum under any of the above conditions. Bergapten (5-methoxypsoralen) was identified in the extract of the fresh celery-S. sclerotiorum system. Xanthotoxin and bergapten were produced only in the rotted areas. Coinciding with the results of Perone et al. (6), neither was found in healthy portions of the same stalk.

From this study, it seems that xanthotoxin and bergapten production requires the active metabolism of celery and of S. sclerotiorum jointly. It was not possible to cause the mold to produce these compounds on nutrient media even when fortified with 30% filter-sterilized celery juice. No xanthotoxin or bergapten was produced by S. sclerotiorum growing on nonmetabolizing (steamed) celery.

The effect of temperature within the range 5 to 30 C on the production of xanthotoxin was studied by infecting fresh celery with S. sclerotiorum and allowing it to grow until each rotted area on the celery stalks was about 4-cm long. The rate of growth of the mold (Table 2) was judged by the number of days of incubation necessary to produce approximately equal rotted areas. No infection could be observed within a month or more at 15 C or below. When celery was held at temperatures higher than 15 C, the rate of infection increased as the temperature increased. Areas that required 8 days to become diseased at 20 C took only 5 days at 30 C. Xanthotoxin production, however, decreased between 20 and 30 C indicating that growing time was a more important factor than the area diseased. Maximal xanthotoxin production was 320 µg of xanthotoxin per g of rotted celery.

TABLE 2. Effect of temperature on xanthotoxin production

| Temp | Dry wt of rotted area (g) | Xanthotoxin produced (µg) | Xanthotoxin (µg/µg of rotted celery) | Days incubation for equal rotted area |
|------|--------------------------|---------------------------|-------------------------------------|-------------------------------------|
| 5 C  | None                     | 490                       | 320                                 | 30a                                 |
| 10 C | None                     | 380                       | 216                                 | 30a                                 |
| 15 C | None                     | 150                       | 83                                  | 5                                   |
| 20 C | 1.5                      |                           |                                     |                                     |
| 25 C | 1.8                      |                           |                                     |                                     |
| 30 C | 1.8                      |                           |                                     |                                     |

* No visible infection.
toxin per g (dry weight) of rotted celery at 20 C. This declined to only 83 µg/g at 30 C. The relative humidity was 100% in every case.

The growth of *S. sclerotiorum* was accompanied by the appearance of a characteristic blue-fluorescing TLC spot at *R*<sub>s</sub> = 0.56 in the benzene-ether (18:7) developing system. This compound appeared when *S. sclerotiorum* grew on nutrient media, celery extracts, non-metabolizing (steamed) celery, and every diseased vegetable except rotted celery where xanthotoxin and bergapten were produced. It was not determined whether celery utilizes this compound from the mold in the production of xanthotoxin or bergapten.

The ability of *S. sclerotiorum* to produce xanthotoxin and bergapten on several foods other than celery was determined. Although lettuce, cabbage, green beans, and strawberries were easily infected and supported growth well, xanthotoxin, bergapten, and 4,5',8-trimethylpsoralen were not produced. Carrots, sweet potatoes, artichokes, oranges, cucumbers, and turnips were less easily diseased, but even after allowing longer incubation to obtain sufficient mold growth, neither xanthotoxin, bergapten, nor trimethylpsoralen could be detected. A significant infection of *S. sclerotiorum* could not be obtained on potatoes. These limited findings failed to demonstrate that *S. sclerotiorum* infection of food commodities other than fresh celery presented a phototoxicological hazard.

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