Isolation of AM-Toxin I, A New Phytotoxic Metabolite from Alternaria mali

T. Ueno, Y. Hayashi, T. Nakashima, H. Fukami, S. Nishimura, K. Kohmoto, and A. Sekiguchi

Research Associate, Graduate Student, Graduate Student, and Professor respectively, Pesticide Research Institute, Kyoto University, Kitashirakawa Sakyo-ku Kyoto Japan; Professor and Associate Professor respectively, Department of Agriculture, Tottori University, Koyama Tottori Japan; and Associate Plant Pathologist, Nagano Horticultural Experimental Station, Susaka Nagano, Japan.

The authors are grateful to Miss M. Uemoto, Pesticide Research Institute, Kyoto University, for her technical assistance on the basic parts of the experiments reported here.

ABSTRACT

A host-specific toxin (AM-toxin I, $C_{23}H_{31}N_3O_6$) has been isolated from culture filtrates of *Alternaria mali*, the causal agent of blotch of apple. This compound produces veinal necrosis on susceptible leaves of apple in very low concentrations, and has the same host specificity in phytotoxic action as the causal fungus.

Phytopathology 65:82-83

Additional key words: Alternaria blotch of apple.

Alternaria mali Roberts causes blotch on the leaves and fruits of apple, especially susceptible cultivars such as "Indo" and "Delicious" common in Japan (2,4). Necrosis resembling the characteristic symptom of the disease in nature can be reproduced not only by artificial inoculation of the pathogen, but also by placing a droplet of its culture filtrate on the leaves. This suggests that the necrotic symptom of apple leaves with *A. mali* may be due to a toxin produced by the fungus (1,3). Several organic solvent extracts (ethyl acetate, chloroform, or dichloromethane) of culture filtrates show toxic activity even in a dilute solution, and on thin-layer chromatograms give a complex medley of spots, one of which can induce veinal necrosis on apple leaves. This paper deals with the isolation of the major one of these active principles, which is tentatively named "AM-toxin I", and shows toxicity to the cells in the vascular system of susceptible leaves.

The production of toxins in the culture medium and their isolation and purification were monitored by the following methods; (i) The sample solution was prepared by successive 10-fold dilutions of ethyl acetate. (ii) Ten μl of the sample solution was then spotted on a square silica gel thin-layer plate (1 × 1 cm, Kieselgel G nach Stahl, 0.25-mm thick, E. Merck) and allowed to air-dry to remove the solvent. The silica gel containing the sample was then scraped off the glass plate and placed in a small circle on the lower side of a fresh apple leaf (susceptible cultivar, Indo) which had been removed from a tree just before use and placed, upper side down, on moist cotton in a petri dish. (iii) The silica gel on the leaf was wetted with 100 μl of distilled water and incubated in a moist chamber at 28 C. The leaf was examined for the induction of veinal necrosis after 18 - 20 hours.

Results of biological assays showed that the culture medium attained maximal activity when *A. mali* (strain number I-716) was cultivated in Richards' medium in still culture at 28 C for 12 days. The culture medium (20 liters) which showed maximal toxic activity was filtered, extracted with an equal volume of ethyl acetate at pH 6.0, which was evaporated to dryness under reduced pressure and yielded an active brown residue (1.7 g). The isolation of the active principle was undertaken through following steps: the residue was redissolved in deionized water (300 ml), and washed with cyclohexane (150 ml × 2) to remove lipid impurities (0.29 g) and extracted with dichloromethane (150 ml × 2) to give on evaporation an oily residue (0.83 g), which was then dissolved in 5 ml of chloroform-ethyl acetate (7:3, v/v) mixture and subjected to column chromatography with silicic acid

(Mallinckrodt, 100 mesh, 35 g). The fractional elution was carried out with chloroform containing 30-40% ethyl acetate. The fractions containing AM-toxin I were combined and evaporated in vacuo to give a syrup (271 mg), which was purified with preparative thin-layer chromatography (Kieselgel GF_{254} nach Stahl Type 60 1-mm thick, E. Merck) by developing with ether-benzene-ethanol (3:2:1, v/v/v) mixture. The active fraction on the silica gel plate was scraped off and extracted with ethyl acetate. Evaporation of the solvent gave a colorless solid (41.6 mg), which was recrystallized from ethyl acetate to give AM-toxin I as fine needles, mp 192-193 C, showing one spot detected by ultraviolet light (254 nm) and iodine vapor on a thin-layer chromatography plate (Kieselgel GF_{254} nach Stahl Type 60, 0.25-mm thick, E. Merck) in the following solvent systems; $R_f = 0.50$ (Rhodamine B, a pilot dye, $R_f = 0.38$) with benzene-acetone (2:1, v/v) mixture, and $R_f = 0.25$ (Benzidine, a pilot dye, $R_f = 0.59$) with cyclohexane-ethyl acetate-*n*-propanol (15:10:1, v/v/v) mixture. AM-toxin I is soluble in water, alcohol, chloroform, and acetone, and practically insoluble in hexane, ether and benzene. By elemental analysis the molecular formula for AM-toxin I was concluded to be $C_{23}H_{31}N_3O_6$ (found: C, 61.99; H, 6.43; N, 9.52 percent; calculated: C, 62.00; H, 7.01; N, 9.43 percent) and by high-resolution mass spectrometry (the measured molecular weight peak was 445.2240 corresponding to the calculated value for the molecular formula 445.2211).

The results of biological assay showed that the crystalline AM-toxin I had a host-specific phytotoxic property resembling that of the causal fungus when it was applied according to the method described above. The threshold concentration which induced veinal necrosis on the leaves of a susceptible cultivar Indo was 0.002 μg/ml, while that on a resistant cultivar Jonathan was 200 μg/ml. When the susceptible apple cuttings were dipped in the toxin solution, veinal necrosis on the leaves was also observed after 18 hours. With this assay method, the threshold concentration was 0.0001 μg/ml on Indo, and 1.0 μg/ml on Jonathan. These results show clearly that the activity of AM-toxin I is specific for apple cultivars which are susceptible to *A. mali*.

Studies on the structural elucidation of AM-toxin I will be reported in the near future. Besides, careful examination of the culture filtrate of a highly pathogenic *A. mali* isolate, produced at different times, has revealed the existence of the five other compounds, showing same host-specific toxicity as AM-toxin I. Isolation and purification of these compounds is currently in progress.

LITERATURE CITED

1. RENBUTSU, Y., S. NISHIMURA, K. KOHMOTO, T. UENO, and A. SEKIGUCHI. 1972. Isolation of host-specific toxins from culture filtrates of Alternaria mali. Ann. Phytopathol. Soc. Jap. 38:194-195 (Abstr.).
2. SAWAMURA, K. 1962. Studies on spotted diseases of apples. I. Causal agent Alternaria blotch. Bull. Tohoku Agric. Exp. Stn. (Japan) 23:163-175.
3. SAWAMURA, K. 1966. Studies on spotted diseases of apples. VI. On the host-specific toxin of Alternaria mali Roberts. Bull. Hortic. Res. Stn. (Japan) Ser. C 4:43-59.
4. SAWAMURA, K., and H. YANASE. 1963. Studies on spotted diseases of apples. II. On Alternaria sp., causal organism of Alternaria blotch and Japanese name of the disease. Bull. Hortic. Res. Stn. (Japan), Ser. C 1:77-94.