# THE ISOLATION AND IDENTIFICATION OF TRICHOTHECENE METABOLITES FROM A PLANT PATHOGENIC STRAIN OF *MYROTHECIUM RORIDUM*[1]

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*Myrothecium roridum* Tode ex Fr. and, less frequently, *Myrothecium verrucaria* (Alb. & Schw.) Ditm. ex Fr. are reported to be root pathogens of red clover and alfalfa (1) as well as leaf and stem pathogens of a number of plants including tobacco, coffee, cotton, gloxinia, snapdragons, and lotus (2-7). Recently, Bruton (8) found that *M. roridum* also attacks muskmelon (*Cucumis melo* L.), causing fruit losses up to 30%, and although all parts of the plant were infected, *M. roridum* most often occurred on the fruit of muskmelons. The genus *Myrothecium* is also known to produce trichothecene metabolites that exhibit activity against a wide number of organisms and can cause phytotoxic, cytotoxic, and cytostatic effects as well (9). This study reports trichothecene production by a pathogenic strain of *M. roridum* from muskmelon, and the absorption, translocation, and metabolism of trichothecenes by muskmelon seedlings.

## EXPERIMENTAL

ISOLATION AND CULTURE OF FUNGUS.—The strain of *M. roridum* was isolated from diseased, immature muskmelon fruit (*C. melo*) in the spring of 1984, identified by Dr. M. Dunn, USDA, Beltsville, Maryland, and a reference culture sent to the Americcan Type Culture Collection, Rockville, Maryland. The isolate was maintained on potato-dextrose agar slants at 24° under laboratory light conditions, which resulted in greenish-black masses of conidia 7-10 days after transfer. A conidial suspension in sterile distilled $H_2O$ (0.1) ml was used to inoculate subsequent cultures for trichothecene production. The isolate was grown for 3 days in 250-ml Erlenmyer flasks containing 50 ml of corn steep medium (corn steep, 10 ml; glucose, 15.6 g plus $H_2O$ to equal 1 liter) and the mycelial pellets transferred to 1-liter Erlenmyer flasks containing 500 ml of a medium composed of $NH_4H_2PO_4$, 1.0 g; $K_2HPO_4$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 5.0 g; sucrose, 40.0 g; and glycerol, 10.0 g plus $H_2O$ to equal 1 liter. After 5-7 days on a rotary shaker (150 rpm) at 24° under normal laboratory light conditions, the mycelium and filtrate were separated by filtration; the mycelium was extracted with 15 ml MeOH, and the filtrate was extracted with 50 ml of EtOAc. The extracts were concentrated by flash evaporation and the residues combined. The residue was redissolved and spotted onto Whatman precoated K6F silica gel tlc plates along with trichothecene standards. The developing solvent system was 5% MeOH in $CHCl_3$, and the trichothecenes were detected using short wavelength uv light and vanillin spray (11).

ISOLATION AND CHARACTERIZATION OF TOXINS.—The crude extract (210 mg) was subjected to chromatography (10 g of 13-25 silica gel) under vacuum (filtration chromatography), elution first with 30 ml of $CH_2Cl_2$ followed by 50 ml of 10% MeOH in $CH_2Cl_2$. The latter fraction was concentrated to give 100 mg of a yellow oil that was applied to a 2-mm silica gel circular plate; this plate was developed (2-8% MeOH-$CH_2Cl_2$) on a chromatatron (Harrison Research Laboratory, Model 7924). Bands corresponding to those of roridin E(**4**), trichodermadienediols A(**5**), and B(**6**), roridin L-2(**7**), and 16-hydroxyroridin L-2 in order of increasing retention time, were collected. Subjection of each of these fractions to preparative hplc (Gilson Model 302, Supelco LC-Si 25 cm × 10 mm column, 50-100% EtOAc-hexane) gave 2.1 mg of roridin E(**4**), 3.5 mg of trichodermadienediol A(**5**), 6.0 mg of
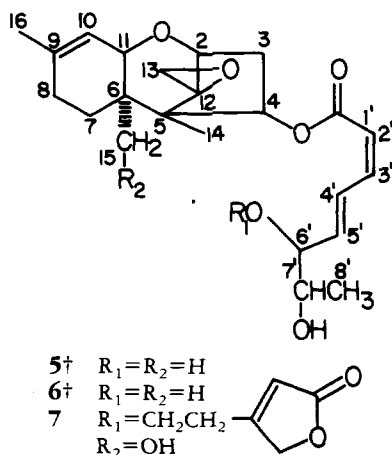
trichodermadienediol B(**6**), 5.1 mg of roridin L-2(**7**), and 4.7 mg of 16-hydroxyroridin L-2. These compounds exhibited identical pmr spectra (200 MHz, IBM WP-200 SY instrument), tlc, and hplc behavior with those of the authentic compounds.

TRANSLOCATION STUDIES.—Six seedlings of *C. melo* grown in the greenhouse and approximately 6 weeks old were placed in an aqueous solution containing roridin A(**3**). The solution was prepared by the addition of 25 mg of roridin A in 2.5 ml EtOH to 50 ml of distilled $H_2O$ (10 $\mu$M $CaSO_4$). Only the root systems were immersed, and after 25 h, the seedlings were removed, the roots washed with tap $H_2O$ and separated from the upper portion of the seedlings. The plant material was freeze-dried and extracted with EtOAc. The crude extracts were taken up in $CH_2Cl_2$ and filtered through activated silica gel. The silica was washed with 20% MeOH in $CH_2Cl_2$ and the eluent analyzed for trichothecenes using tlc and hplc as described earlier (15). Naturally infected areas (each 4 cm in diameter, one per fruit) were excised from five green fruits, extracted with EtOAc (100 ml) and analyzed by the same method.

## DISCUSSION

*M. verrucaria* and *M. roridum* are reported producers of the potent cytotoxic macrocyclic trichothecenes (10). Recently, *M. verrucaria* was shown to produce trichoverroids (e.g., **5** and **6**) which are believed to lie along the biosynthetic pathway to the macrocyclic roridin and verrucarin trichothecenes (11). The principal trichothecene metabolites of *M. verrucaria* and *M. roridum* are usually verrucarins A(**1**) and J(**2**) and roridins A(**3**) and E (and/or *iso*E,

1   $R_1, R_2=O; R_3=-CHOHCHCH_2-$
2   $R_1, R_2=O; R_3=-CH=CCH_2-$
3   $R_1,=H; R_2=CHOHCH_2;$
    $R_3=-CHOHCHCH_2-$
4   $R_1,=H; R_2=-CHOCH_2;$
    $R_3=-CH=CCH_3-$

5†   $R_1=R_2=H$
6†   $R_1=R_2=H$
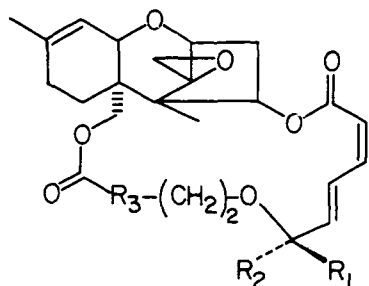7    $R_1=CH_2CH_2-$
     $R_2=OH$

†For **5** and **6**, C-7′ differ in configuration (11).

**4**) (10,13). Under our culture conditions, this strain of *M. roridum* produced very little of the macrocyclic trichothecenes but instead produced mainly the trichoverroids: trichodermadienediols A(**5**) and B(**6**), roridin L-2(**7**), and 16-hydroxyroridin L-2. In another study (12), we found that the strain of *M. roridum* [81-131] which is a pathogen on *Aglaonema* sp. also produces principally trichoverroids (**5**, **6** and **7**) with little production of the macrocyclic trichothecenes.

Trichothecene production by *M. roridum*, under the conditions of our study, was low compared to *M. verrucaria* (13). The level of trichodermadiendiols A(**5**) and B(**6**) produced was approximately 10 mg-liter. Roridin L-2(**7**) and 16-hydroxyroridin L-2 were produced at a level of approximately 5 mg/liter, and less than 5 mg/liter of roridin E(**4**) was produced.

Within 24 h of exposure to roridin A(**3**), muskmelon seedlings were able to absorb, translocate, and metabolize roridin A to 8β-hydroxyroridin A, whereas seedlings not exposed to roridin A contained no trichothecenes; however, we were unable to detect trichothecenes in naturally infected plant material, and we can only speculate as to whether the trichothecenes produced by *M. roridin* are involved in the pathogenicity of this fungus. In 1969, Cunfer and Lukezic

(14) suggested that an unidentified metabolite of *M. roridum* was involved in the establishment of this pathogen on red clover, and the same situation may exist with this isolate of *M. roridum* and muskmelons. However, what may be more important is the ability of plants to absorb and accumulate significant levels of mycotoxins that may prove toxic if consumed by animals or people. For example, we reported in 1981 that *Baccharis megapotamica* plants are able to absorb, translocate, and metabolize trichothecenes produced by soil fungi (15). This suggests that the high levels of baccharinoid trichothecenes normally found in *B. megapotamica*, which causes these plants to be toxic to animals when consumed, may have been absorbed from the soil where they were being produced by *M. roridum*. The role of microbially produced trichothecenes in the growth and response of plants to pathogenic microorganisms is under investigation.

## ACKNOWLEDGMENTS

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