

A Toxin from *Myrothecium roridum* and its Possible Role in *Myrothecium* Leaf Spot of Red Clover

B. M. Cunfer and F. L. Lukezic

Pennsylvania Agricultural Experiment Station and United States Regional Pasture Research Laboratory, University Park 16802. Present address of senior author, Department of Plant Pathology, Washington State University, Pullman 99163.

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ABSTRACT

A phytotoxic metabolite was purified and partially characterized from the fluid associated with sporodochia of *Myrothecium roridum* grown on autoclaved red clover leaves. The compound, which is stable for long periods of time, produces necrosis on red clover foliage and other species. It is neutral, aliphatic, nonproteinaceous, and contains a carbonyl bond. Isolates of *M. verrucaria* that were tested also produce the toxin.

Based on its infrared spectrum and phytotoxic properties, the compound is similar to necrocitin, and antifungal, phytotoxic metabolite from *M. roridum* culture filtrates.

Though not directly linked to pathogenicity, the toxin aids in symptom development by creating infection courts on which spores may germinate and penetrate the foliage. *Phytopathology* 60:341-344.

Myrothecium leaf spot of red clover begins as small, dark, water-soaked lesions that become necrotic and extend to both leaf surfaces. The lesions initially are circular spots, but become irregular and coalesce as they enlarge. The lesions are dark brown to black, but may occasionally form concentric lighter and darker brown rings, giving a target-spot effect. Slight chlorosis may sometimes be seen around lesions, and some lesions may be restricted by leaf veins (4). However, when leaves of red clover (*Trifolium pratense* L.) are inoculated with conidial suspensions of *Myrothecium roridum* Tode ex Fries, numerous minute, necrotic spots often appear within 24 to 48 hr. Many of these do not enlarge, and no fungal growth is associated with them, suggesting that a phytotoxic compound produced by the fungus might be responsible. This hypothesis is supported by reports that materials toxic to both plants and animals are associated with cultures of *M. verrucaria* and *M. roridum*. In 1946, Brian & McGowan (3) isolated the compound glutinosin from *M. verrucaria* Ditmar which prevented germination of spores of several fungus species. Since this discovery, *M. roridum* and *M. verrucaria* have been investigated extensively as possible sources of fungicidal materials. More than twenty biologically active metabolites have been identified from the two organisms.

Brian & McGowan (3) reported unidentified materials causing severe dermatitis on humans in the ethanol mother liquors of cultures of *Metarrhizium glutinosum* (*M. verrucaria*). In 1955, Bowden & Schantz (2) identified one of these dermatitic compounds as being weakly acidic with an empirical formula of $C_{15}H_{22}O_4$.

No other metabolites from *Myrothecium* species were reported until 1961 when Nespiak et al. (12) isolated three metabolites from *M. roridum* culture filtrates. Myrothecin, the major metabolite, and two minor metabolites were isolated by extraction with chloroform, separation chromatography on activated

alumina, and recrystallization. Myrothecin was inactive, but the first minor metabolite and the noncrystalline mother liquors were highly fungicidal. Activated charcoal adsorbed the fungicidal metabolites from the mother liquors. Nespiak also reported that actively growing cultures of *M. roridum* inhibited growth of various fungi, but a suspension of dried mycelium was inactive.

Kishaba et al. (9) identified two compounds from *M. roridum* which inhibited feeding and killed adults and larvae of the Mexican bean beetle, *Epilanchma varivestis*. Bean (*Phaseolus vulgaris*) leaves were immersed in the toxin solutions during feeding tests, but the results do not report any evidence of phytotoxicity.

Harri et al. (8) reported 10 neutral lipophilic metabolites from *M. roridum* and *M. verrucaria* which they named roridins and verrucarins. The compounds were isolated by extraction of culture filtrates with petroleum ether and thin-layer chromatography on Kieselgel G. The compounds were detectable in ultraviolet light and with iodine vapor.

From *M. verrucaria*, Vittimberga (17) identified muconomycin A, later found to be identical to verrucarins A, which shows high antibiotic activity and causes a severe dermatitis on humans. Vittimberga & Vittimberga (18) subsequently isolated muconomycin B from *M. verrucaria*. It is similar to A but has twice its antifungal activity.

Pawar et al. (13) and Pawar & Thirumalachar (14) reported that a compound designated "necrocitin" from culture filtrates of *M. roridum* showed high antifungal but no antibacterial activity. Necrocitin also causes necrosis on bean cotyledons and wilting of tomato (*Lycopersicon esculentum*) shoots. This is the only report of a metabolite of either of these fungi which was investigated for phytotoxic properties. The purpose of this study was to investigate the possible production of a toxin by *M. roridum* and its role in the development of *Myrothecium* leaf spot of red clover.

MATERIALS AND METHODS.—To favor sporulation over the formation of aerial hyphae, the fungus was cultured on autoclaved red clover leaves. The leaves were inoculated by immersion in a spore suspension of *M. roridum* (isolate 535), immediately placed on 2% water agar in petri dishes, and incubated at 30 C in constant light at 150 to 250 ft-c for 3 to 5 days. This technique produced masses of sporodochia on the surface of the leaf (Fig. 1).

Preliminary experiments to determine if the toxin was associated with sporodochial fluid were completed by collecting the fluid in micropipettes. This fluid was sterilized by filtration through a 0.45- μ cellulose membrane filter (Swinney adaptor). Because of limitations in the amount of fluid collected in this manner in later experiments, the fluids were collected from the young sporodochia in small amounts of sterile, distilled water. Spores were removed from the liquid by centrifugation and filtration. Sterility of the extract was determined by placing part of the extract on malt extract agar. The water solution of sporodochial fluid (crude toxin) was stored at 5 C until the time of extraction or for use in bioassay studies. To determine if the toxin was associated with mycelium, cultures of the fungus were grown on malt extract agar. Water extraction was made from these cultures for the bioassay.

To test the ability of the various extracts to cause necrosis, 2 to 3 droplets (approx. 0.1 ml) of the given extract were applied to red clover foliage. To retard evaporation, the plants were covered with a plastic bag for 24 hr. The plants were kept at room temperature in the laboratory for observation.

The controls consisted of washing noninoculated leaves which had been treated in the same manner as the inoculated leaves. Each test solution was placed on three different leaflets.

A series of preliminary tests was completed to determine some of the physical and chemical properties of the crude toxin. The stability of the toxin stored at 5 C and 30 C for 30 days, and after autoclaving, was determined by bioassay. A sample at pH 2 was passed through a column of Rexyn 101 cation exchange

resin and Rexyn 201 anion exchange resin, neutralized and bioassayed to determine if the compound had an electrical charge. Adjustments of pH were made with 0.1 M HCl and 0.1 M NaOH. Another sample was passed through the resins at pH 7. Darco C50 activated charcoal was mixed with the crude toxin, filtered, and the filtrate bioassayed. The possible presence of reducing sugars was tested by using descending paper chromatography in water-saturated phenol in an ammonia atmosphere. The chromatogram was sprayed with ammoniacal silver nitrate and dried at 100 C in an oven (1). The Le Rosen test for aromatic compounds, organic acid test with Nessler reagent, and tests for protein were conducted as described by Feigl (5). Finally, the crude toxin was bioassayed on Ladino clover (*Trifolium repens* L.), alfalfa (*Medicago sativa* L.), and gardenia (*Gardenia veitchii* Hort.).

The phytotoxic factor was isolated and purified by chloroform extraction and thin-layer chromatography. The crude toxin was adsorbed to Darco C50 activated charcoal which was washed with distilled water to remove the nonadsorbed materials. The charcoal was washed three times with each of three portions of chloroform by suction filtration. The combined filtrates were evaporated to dryness.

The residue was redissolved in absolute ethanol, streaked on a Kieselgel G thin-layer plate, and developed with chloroform:methanol (97:3, v/v) following the method of Harri et al. (8). A light fluorescent band at R_F 0.80-0.90 in ultraviolet light was eluted with chloroform, filtered, and evaporated to dryness. The residue was bioassayed.

A sample of the purified toxin in a saturated solution of carbon tetrachloride was analysed by infrared spectroscopy with a Perkin-Elmer 421 spectrophotometer. The path length was 0.015 mm.

Influence of toxin on pathogenicity.—The possible relation of the toxin to pathogenicity was determined by testing the spore-free fluid of each of 15 different isolates of *M. roridum* and *M. verrucaria*. This selection consisted of 5 *M. roridum* and 4 *M. verrucaria* isolates that were not pathogenic, and 5 *M. roridum* and 1 *M. verrucaria* isolates that were pathogenic, to red clover. The sporodochial fluid of these isolates was prepared as previously described and bioassayed on red clover.

The spores of each isolate were washed several times with sterile distilled water by centrifuging and decanting to remove all sporodochial fluid. The washed spores were divided into two portions and prepared as spore suspensions with the addition of distilled water and Tween 20 (polyoxyethylene sorbitan monolaurate). The sporodochial fluid used was obtained by washing 10 densely sporulating cultures of isolate 535 with 20 ml of sterile distilled water. To one 40-ml portion of spore suspension, 1 ml of the filtered toxin was added. The amount of 535 toxin added was equivalent to that present in a normal nonwashed spore suspension. This concentration of toxin was sufficient to produce necrosis on the leaves. The control spores were suspended in sterile water.

Leaflets on red clover plants were inoculated with

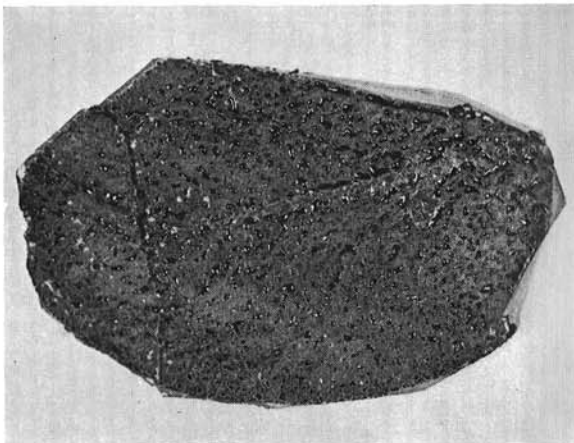


Fig. 1. Sporodochia of isolate 535 (*Myrothecium roridum*) on autoclaved red clover leaf on water agar.

both suspensions and incubated at 30 C with a 16-hr photoperiod for 4 days. Plants were rated for symptoms at the termination of this period. The test was replicated twice and repeated twice.

A regular spore suspension and suspension of washed spores of *M. roridum* isolate 535 were inoculated onto red clover leaflets to determine the relation of toxin to host penetration. One half of the test leaflets were injured by puncture with a sterile teasing needle, while the other half were left intact. At 4-hr intervals, leaves were removed from the plants, decolorized in phenol, and stained with lactophenol-cotton blue. Spore germination was observed directly on the leaves or on epidermal strips at wounded, intact, and toxin-killed tissue.

RESULTS AND OBSERVATIONS.—A toxin causing necrosis on red clover foliage was isolated from the sporodochial fluid of *M. roridum* (isolate 535) by adsorption on activated charcoal, chloroform extraction, and thin-layer chromatography. This necrosis is identical to that observed on foliage 1 day after inoculation with spore suspension. Approximately 1 liter of crude toxin extract yielded 18 mg of purified toxin.

While the toxin does not react to iodine vapor, several nontoxic substances below the toxin on a Kieselgel plate react to iodine vapor.

The toxin is soluble in carbon tetrachloride, chloroform, and absolute ethanol, but only slightly soluble in water. No loss of activity of crude toxin is observed after autoclaving or after 30 days at 5 and 30 C. The toxic factor is not removed by Rexyn 101 and Rexyn 201 ion-exchange resins at pH 7, but activity is lost when passed through these resins at pH 2. Activity is not lost when the crude toxin is adjusted to pH 2 and subsequently neutralized. Activated charcoal also completely adsorbs the toxic factor. Negative tests for reducing sugars, aromatic compounds, organic acids, and proteins were obtained. Sterile mycelial filtrates also produced necrosis when bioassayed. Bioassays of the crude toxin on Ladino clover and alfalfa gave positive reactions, though less intense than on red clover. No necrosis appeared on gardenia.

The location of the peaks of the infrared spectrum

of purified 535 toxin (Fig. 2) is very similar to those of necrocin (Table 1).

Role of toxin in the disease.—The sporodochial fluid of all 15 isolates, pathogenic or not, caused necrosis on the leaves of red clover.

With the toxin added to spore suspensions of the various isolates, necrosis was always seen on the foliage, but only washed spores of isolate 535 with crude 535 toxin added caused typical symptom development. Plants inoculated with washed spores did not develop symptoms or necrosis.

On foliage inoculated with a suspension of unwashed spores, spore germination begins rapidly at wounds, but on healthy tissue no germination was seen until epidermal cells are killed by the toxin. Since the cells of intact tissue were not killed by the toxin for at least 24 hr after inoculation, symptoms begin earlier at wounds. Once an area is killed by the toxin, it will not enlarge unless it is invaded by the fungus. Germination of washed spores occurred only where there were dead or injured cells from wounds.

DISCUSSION.—Based on the infrared spectrum and other tests, the toxic compound is a neutral, aliphatic, nonproteinaceous compound containing at least one carbonyl bond. The toxin does not react to iodine vapor as did metabolites isolated by Harri et al. (8). The infrared spectrum of the compound is nearly identical to that of necrocin isolated from culture filtrates. Mycelial filtrates of isolate 535 grown on malt extract agar also produce necrosis on red clover; however, the role of the toxins in the filtrates in pathogenicity in nature is not known. While the origin of the sporodochial fluid is not known, based on these observations, it is possible that the fluid may be secreted by the conidiophores or other aerial hyphae at the time of sporulation.

The possibility that sporodochial fluid (i.e., toxin) may be splash-dispersed with the spores is well documented by the work of Gregory et al. (7). They showed that water drops striking conidial fructifications of *Nectria cinnabarina* produced splash droplets consisting of a mixture of liquid from both the incident drop and the surface suspension.

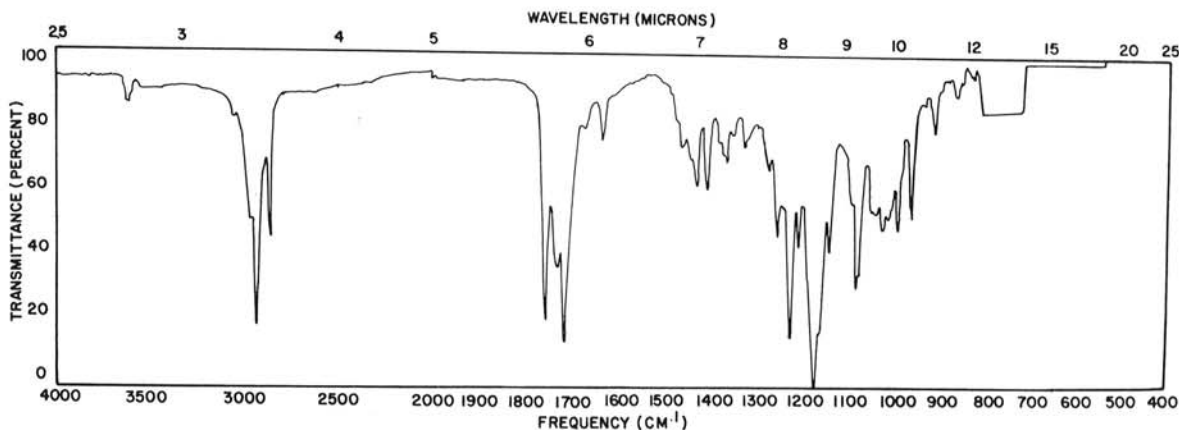


Fig. 2. Infrared spectrum of purified *Myrothecium roridum* toxin in carbon tetrachloride.

TABLE 1. A comparison of the location of peaks (in μ) of the infrared spectrum of necrocitin (12) and purified 535 toxin

Necrocitin	3.37		5.68		5.83	6.10		7.06
535 toxin	3.42	3.50	5.69	5.77	5.82	6.10	6.98	7.10
Necrocitin	7.38			8.55		9.17	10.1	10.3
535 toxin	7.30	7.53	8.13	8.50	8.74	9.20	10.2	10.3

As both pathogenic and nonpathogenic isolates tested produce the toxin, it appears that the ability to produce toxin is not directly linked to pathogenicity. Washed spores alone and washed spores in combination with 535 sporodochial fluid added do not produce symptoms on red clover, with one exception. Isolate 535 produces typical symptoms when its own fluid is present. The toxin may play a secondary role with some additional factor responsible for pathogenicity. If the hyphae are incapable of colonizing living cells, the toxin may provide a substrate of dead or dying cells for the fungus. However, because pathogenic isolates do not produce symptoms on leaves that have tissue killed by 535 toxin, other unexplained factors may govern host colonization.

In the literature, *M. roridum* is reported primarily as a wound pathogen (6, 15, 16, 20). Penetration of unwounded tissue of red clover occurs only where cells have been killed by the toxin. Fergus reported that *M. roridum* on gardenia infected only previously wounded tissue (6). The sporodochial fluid of his isolate, 535 of the present work, does not cause necrosis on gardenia leaves. Other reports in the literature (11, 15), which state that wounds are not required for penetration by *M. roridum*, indicate the possibility of necrosis caused by toxic materials in the sporodochial fluid prior to spore germination.

The definition of the term "toxin" is one that has been discussed for many years in the literature (10, 19). It is helpful to orient the current findings to the observations of other workers. The compound is a toxin as defined by Ludwig (10); it is a product of a microorganism which acts directly on living host protoplasts to influence the course of disease development or symptom expression. It does not meet the stringent requirements for vivotoxins or of pathotoxins (19), in that the pathogen and toxin do not have similar host specificity, nor does the ability to produce toxin vary with pathogenicity.

Therefore, with the present information on this compound, it should be classified under Ludwig's general definition of toxin until additional knowledge is gained about it.

LITERATURE CITED

- BLOCK, R. J., E. L. DURRAM, & G. ZWEIG. 1958. A manual of paper chromatography and paper electrophoresis [2nd ed.] Academic Press, N.Y. 710 p.
- BOWDEN, J. P., & E. I. SCHANTZ. 1955. The isolation and characterization of dermatitic compounds produced by *Myrothecium verrucaria*. J. Biol. Chem. 214:365-372.
- BRIAN, P. W., & J. C. MCGOWAN. 1946. Biologically active metabolic products of the mould *Metarrhizium glutinosum* Pope. Nature 157:334.
- CUNFER, B. M., J. H. GRAHAM, & F. L. LUKEZIC. Studies on the biology of *Myrothecium roridum* and *M. verrucaria* on red clover. Phytopathology 59: 1306-1309.
- FEIGL, F. 1956. Spot tests in organic analysis [5th ed.] Elsevier Pub. Co., N.Y. 616 p.
- FERGUS, C. L. 1957. *Myrothecium roridum* on Gardenia. Mycologia 49:124-127.
- GREGORY, P. H., E. J. GUTHRIE, & MAUREEN E. BUNCE. 1959. Experiments on splash dispersal of fungus spores. J. Gen. Microbiol. 20:328-354.
- HARRI, E., W. L. LOEFFLER, H. P. SIGG, H. STAHELIN, C. STOLL, C. TAMM, & D. WIESINGER. 1962. Verrucarins and roridins, a group of cytostatic high acting antibiotics from *Myrothecium* species. Helv. Chem. Acta 45:839-853.
- KISHABA, A. N., D. L. SHANKLAND, R. W. CURTIS, & M. C. WILSON. 1962. Substances inhibitory to insect-feeding with insecticidal properties from fungi. J. Econ. Entomol. 55:211-214.
- LUDWIG, R. A. 1960. Toxins. p. 315-358. In J. G. Horsfall & A. E. Dimond [ed.] Plant Pathology, an advanced treatise, Vol. II. Academic Press, N.Y.
- MUNJAL, R. L. 1960. A commonly occurring leaf spot disease caused by *Myrothecium roridum* Tode ex Fr. Ind. Phytopathology 13:150-155.
- NESPIAK, A., M. KOCO, & A. SIEWINSKI. 1961. Antibiotic properties of mycelium and metabolites of *Myrothecium roridum* Tode. Nature 192:138-139.
- PAWAR, V. H., P. V. DESHMUKH, & M. J. THIRUMALACHAR. 1965. Necrocitin: A new crystalline antifungal antibiotic and plant toxin. Hindustan Antibiot. Bull. 8:59-63.
- PAWAR, V. H., & M. J. THIRUMALACHAR. 1966. Necrocitin as a wilt toxin (antifungal antibiotic from *Myrothecium roridum*). Hindustan Antibiot. Bull. 8:126-128.
- PRESTON, N. C. 1935. The parasitism of *Myrothecium roridum* Tode. Brit. Mycol. Soc. Trans. 20:242-251.
- STEVENSON, J. A., & L. P. MCCOLLOCK. 1947. *Myrothecium* as a tomato rot organism. Plant Dis. Repr. 31:147-149.
- VITTIMBERGA, B. M. 1963. The Myconomycins. I. Studies on the structure of Myconomycin A, a new biologically active compound. J. Org. Chem. 28:1786-1789.
- VITTIMBERGA, J. S., & B. M. VITTIMBERGA. 1965. The muconomycins. II. Muconomycin B, a new biologically active compound. J. Org. Chem. 30:746-748.
- WHEELER, H., & H. H. LUKE. 1963. Microbial toxins in plant disease. Annu. Rev. Microbiol. 17:223-242.
- WILHELM, S., W. GUENSCH, & K. F. BAKER. 1945. *Myrothecium* crown and stem canker of greenhouse snapdragons in Colorado. Plant Dis. Repr. 29:700-702.