Phytotoxicity and Pathogenicity of *Fusarium roseum* to Red Clover

F. Blain, M. Bernstein, S. Khanizadeh, and S. A. Sparace

First and last authors: Plant Science Department, Macdonald College of McGill University, 21111 Lakeshore Road, Ste. Anne de Bellevue, Quebec H9X 1C0. Second author: Merck Frosst Canada, Inc., P.O. Box 1005, Point Claire, Quebec H9R 4P8. Third author: Agriculture Canada, 430 Gouin Blvd., St.-Jean-Sur-Richelieu, Quebec J3B 3E6.

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ABSTRACT

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Extracts of culture filtrates from *Fusarium roseum* are phytotoxic to germinating red clover (*Trifolium pratense*) seeds. All isolates tested yielded phytotoxic extracts composed of several phytotoxic components. Extracts from the most pathogenic isolate showed only slight host specificity for susceptible red clover seedlings; cucumber (*Cucumis sativus*) and timothy (*Phleum pratense*) were most resistant at the lowest concentrations tested. Phytotoxic fractions contain primary amino acids and secondary N-substituted amino acids. Preparative thin-layer chromatography yielded two major phytotoxic fractions: a crude crystalline material containing valine residues and an oil containing valine, isovaleric acid, and aromatic phenyl moieties. These phytotoxins resemble the cyclodepsipeptide antibiotics produced by other pathogenic fungi and by other *F. roseum* isolates.

Additional keywords: crown and root rot of clover.

Fusarium roseum Link:Fr. is a major causal agent of root and crown rot of red clover (*Trifolium pratense* L.) (8,13). The pathogen is known to produce a variety of biologically active substances in pure culture, including resorcyclic acid lactone (zearalenone) mycotoxins (9,15), trichothecene mycotoxins (5,6,15), and enniatin cyclodepsipeptide antibiotics (3,15). Although these substances often have phytotoxic properties, phytotoxic metabolites produced by *F. roseum* and their possible roles in the root and crown rot disease of red clover have not been thoroughly investigated. The objectives of this investigation were to examine and compare the toxicity of culture extracts and pathogenicity of several isolates of *F. roseum* in the crown and root rot disease of red clover and characterize the biological and chemical nature of the phytotoxins produced.

MATERIALS AND METHODS

Plant and fungal material. Seeds of red clover cultivars Arlington and Florex and timothy (*Phleum pratense* 'Richmond') were obtained from Otto Pick and Sons Seeds Ltd., St-Hyacinthe, Quebec. Seeds of alfalfa (*Medicago sativa* 'WL316') were obtained from Semico Inc., Ste-Rosalie, Quebec. Anchor alfalfa seeds were obtained from Oseco Inc., Brampton, Ontario. Seeds of Cherry Belle radish (*Raphanus sativus*), Heinz 1350 VF tomato (*Lycopersicon esculentum*), Grand Rapids lettuce (*Lactuca sativa*), Marketmore cucumber (*Cucumis sativus*), and Processor II carrot (*Daucus carota*) were all obtained from Stokes Seeds Ltd., St. Catharines, Ontario. Unless otherwise specified, seeds of red clover cultivar Arlington were used throughout this investigation. Cultures of *F. roseum* isolates 766 and 5215 (Avenaceum), 927 and 959 (Acumenatum), and 814 (undefined) were obtained from K. T. Leath, USDA, University Park, PA. For most studies described here, isolate 5215 was used alone, except when compared with other isolates.

Reagents. Standard laboratory reagents were obtained from local suppliers. L-Valine and *N*-methyl-DL-valine were obtained from Sigma (St. Louis, MO). Whenever possible, all other dry reagents and organic solvents were analytical reagent grade or better. Difco Bacto agar and Bacto potato-dextrose agar were used for solid culture media. UV-indicating silica gel thin-layer chromatography plates (20 × 20 cm; 0.25 mm thick) were obtained from Brinkmann Instruments, Rexdale, Ontario. Except where otherwise mentioned, double-distilled water was used throughout this investigation.

Growth of fungi. Stock cultures of *F. roseum* were maintained under refrigeration at 4 C on potato-dextrose agar supplemented with 2.5 g of KNO_3 and 1 g of KH_2PO_4 per liter. When necessary, stock cultures were subcultured onto the same media in petri dishes and incubated at 25 C. *F. roseum* was grown in a liquid medium containing 50 g of sucrose, 1 g of Difco yeast extract, 3 g of NH_4NO_3, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 27.8 mg of $FeSO_4 \cdot 7H_2O$, and 37.3 mg of Na-EDTA per liter of water. The medium, 100 ml per 250-ml Erlenmeyer flask, was inoculated with a 2- × 2-mm square of actively growing mycelium and adhering agar from appropriate isolates and incubated in a rotary shaker/incubator for 5 days in darkness at 25 C and 150 rpm. The pathogenicity of the five isolates of *F. roseum* in causing crown rot was verified essentially as described by Leath and Kendall (8), except that disease was allowed to develop for 10 wk.

Liquid culture harvest and preparation of crude extracts. Five-day-old cultures were vacuum-filtered through filter paper placed in a Büchner funnel. After fresh weight measurements, mycelial pads were dried at 50 C for 24 hr, and dry weights were then determined. The mycelium then was discarded or kept for mycelium extraction. Culture filtrates were adjusted to pH 3 with HCl, transferred to a separatory funnel, and extracted three times with approximately 65 ml of ethyl acetate. The organic phases were pooled and dried on a rotary evaporator at 35 C. The resulting crude extract was redissolved in 1 ml of methanol for each 100 ml of extracted filtrate (the equivalent of one standard culture flask yielding 40–50 mg of crude extract) and stored under nitrogen at −20 C. Control extracts were obtained by similarly extracting liquid medium of uninoculated flasks. When necessary, the dried mycelium was homogenized with a mortar and pestle in 35 ml of methanol. The mycelial homogenate was clarified by centrifugation at 1,000 *g* for 30 min. Mycelial pellets were further extracted by twice being mixed with 125 ml of methanol for 2 hr and being recentrifuged. The pooled supernatants were centrifuged for 1 hr and passed through a Millipore filter (0.45 μm) to clarify the extract. During drying of the pooled supernatants, an insoluble gumlike fraction formed that would not redissolve in methanol

or chloroform. The gum fraction was discarded, and the chloroform and methanol extracts of the insoluble gum were pooled, centrifuged for 30 min, drawn off, dried, and redissolved in methanol.

Seedling germination/elongation bioassays. Seedling bioassays were performed in culture filtrates directly, in extracts of filtrates or mycelium, and with chromatography fractions of extracts. All extract concentrations were expressed as a fraction of the original culture volume from which it was derived. An extract dilution series was obtained by drying 200 μl of redissolved extract in a test tube and dispersing it in 10 ml of water with vortexing and sonication. This yielded a concentration of extract equal to twice that found in the original culture filtrate (2×). Further dilutions of the 2× extract with water yielded concentrations of 1×, 0.50×, 0.25×, and 0.125×. Dilutions of mycelium extracts were prepared in a similar fashion. Culture filtrates were diluted with water.

Seeds were imbibed by soaking with bubbling aeration in water for 6 hr at room temperature. Twenty-five imbibed seeds were placed in each treatment dilution for 30 min at room temperature, after which seeds and the treatment solution were transferred onto 9-cm filter paper disks placed in a glass petri dish. Plates were covered and the seeds were allowed to germinate in an incubator in darkness at 24 C for 2 days. For each experiment, three dilution series were produced, each with 25 seeds per treatment dilution. Seeds treated with water only or redissolved extracts of uninoculated culture medium were used as controls. After the incubation period, the length of the hypocotyl of each seedling was measured. All experiments were factorials in a completely randomized design.

Thin-layer chromatography. Thirty-five to fifty microliters of crude filtrate extracts was dried on 1.5-cm-wide thin-layer chromatography (TLC) lanes. In the case of preparative TLC, lanes were typically 14–18 cm wide and, depending on the purity of the extract, the equivalent of 35–7,200 μl of original sample extract was plated for each 1.5 cm of lane width. All plates were developed with hexane/ethyl acetate/95% ethanol (70:20:10, v/v), until the solvent front had traveled approximately 14 cm. Zones of interest were identified under short- and long-wave UV light. When necessary, the silica gel from each of the zones of interest was scraped off the plate. The silica gel was then eluted three times with methanol (approximately 3 ml of methanol per milliliter of dry silica gel), and the eluates from each zone were pooled, centrifuged (1,000 *g*) for 30 min, dried, and redissolved in methanol. Eluates of silica gel from unused TLC plates were used as controls.

Column chromatography. Crude extracts of culture filtrates pooled from approximately 13 standard culture flasks (547 mg of dried extract) were redissolved in 3.5 ml of hexane/ethyl acetate/95% ethanol (50:25:25, v/v) and applied to a 2- × 50-cm column containing 40–140 mesh silica gel in hexane/ethyl acetate/95% ethanol (72:18:9, v/v) and a final bed volume of 100 ml. The column was then sequentially eluted at the rate of 2.5 ml/min in a stepwise fashion with 99 ml of each of the following solvent mixtures (v/v) to generate the corresponding fractions I–VII: I = hexane/ethyl acetate/95% ethanol (72:18:9); II = hexane/ethyl acetate/95% ethanol (58:27:14); III = hexane/ethyl acetate (2:1); IV = hexane/ethyl acetate (1:2); V = 100% ethyl acetate; VI = ethyl acetate/methanol (2:1); VII = ethyl acetate/methanol (1:2); and fraction VIII was eluted with 200 ml of 100% methanol. Column fractions were dried as described earlier and redissolved in 3.5 ml of methanol.

Qualitative *N*-methylamino acid determination. The method used for qualitative *N*-methylamino acid determination was adapted from that of Rosen (10) as modified by Audhya and Russell (1) and relies on the differential reactivity of primary and secondary *N*-methylamino acids with ninhydrin. Primary amino acids react with ninhydrin within 15 min, while secondary amino acids require up to 2 hr for full color development (1). Thus, only samples containing secondary amino acids show an increased ninhydrin reaction (i.e., increased absorbance at 575 nm when short and long reaction times are compared). The validity of the procedure was verified with known standards of L-valine and *N*-methyl-DL-valine. Four replicates each of 15–35-μl aliquots of crude filtrate extracts or chromatography fractions were dried in screw cap tubes, redissolved in 0.5 ml of HCl/glacial acetic acid (1:1, v/v), and hydrolyzed at 110 C for 24 hr. After hydrolysis, the mixture was dried under nitrogen, and the residue was redissolved in 1 ml of water and analyzed for amino acids as described above. The ninhydrin absorbance of two replicates of each sample was measured at 15 min and then at 2 hr for the remaining two replicates, and the fold increase in absorbance was calculated.

NMR spectroscopy and statistical analyses. Nuclear magnetic resonance (NMR) analyses of two partially purified phytotoxic components were performed in deuterated acetone or chloroform with a Bruker AM 300 NM spectrometer, operating at 300 MHz in the Fourier transform mode. All experimental data were analyzed with PC-SAS. Except for the pathogenicity test, bioassay experiments were performed three times. Chemical analyses were performed once or twice. Data from representative experiments are presented.

RESULTS AND DISCUSSION

Pathogenicity and virulence characteristics of *F. roseum*. All *F. roseum* isolates tested were pathogenic. Typical lesion characteristics caused by isolates 5215 and 766 compared with controls are shown in Figure 1. All isolates caused either a slight to intense browning or a blackening and necrosis of the central taproot tissues. Depending on the isolate, the lesions extended from the cut inoculation surface into the crown. Isolate 5215 was extremely virulent and caused significantly longer average vertical lesion lengths than the other isolates. Isolate 766 caused the shortest average lesion length (Table 1), although the length did not differ significantly from lesions caused by isolates 814, 927, and 959. In general, isolates causing greater lesion lengths caused the greatest discoloration of plant tissue, with isolate 5215 causing extensive blackened necrotic regions and isolate 766 and the control treatments showing little or no tissue discoloration. The results obtained here are generally in agreement with those of other workers who used these and other isolates of the pathogen in other pathogenicity tests (7,8,13). However, lesion lengths for isolate 766 and the uninoculated cut control are approximately 0.5 cm greater than those reported by Leath and Kendall (8). This is most likely attributable to the greater length of time used for disease development in this study.

Localization and recovery of phytotoxicity in liquid cultures. Unextracted filtrates from 5-day-old cultures were extremely toxic to germinating clover seeds. However, the phytoxicity of fungal products in filtrates was heavily masked by effects of the culture medium, because eightfold dilutions of culture filtrates and uninoculated control filtrates inhibited seedling germination and elongation by 78 and 50%, respectively. Generally, approximately 75% of the phytotoxic activity of culture filtrates was recovered in ethyl acetate extracts of the acidified filtrate. Approximately 60% of the total phytotoxicity from the mycelium plus culture fluids was recovered in the filtrates.

Comparative phytotoxic activities of crude extracts. The five isolates of *F. roseum* were compared for their yield of crude phytotoxic material. On the average, isolates 766, 814, 927, 959, 5215, and the uninoculated control filtrates yielded 34.7 ± 1.3, 38.3 ± 3.1, 52.3 ± 7.1, 52.4 ± 3.4, 50.9 ± 3.9, and 3.4 ± 0.4 mg, respectively, of crude extract per standard culture flask. All such extracts were phytotoxic. The mathematical expression $Y = [\log(aC + b)]^{-1}$, where Y = average seedling length, C = relative extract concentration, and a and b are optimum constants determined by the statistical analysis program, served as a good model for the observed effects. Values for variables a and b ranged from 0.072 to 0.273 and 0.990 to 1.080, respectively. R^2 values for equations ranged from 0.60 for isolate 959 to 0.88 for isolate 5215. At the lowest concentrations tested (0.125×), filtrate extracts from isolate 959 and 5215 were significantly the most phytotoxic (average seedling lengths were 21.3 ± 4.9 and

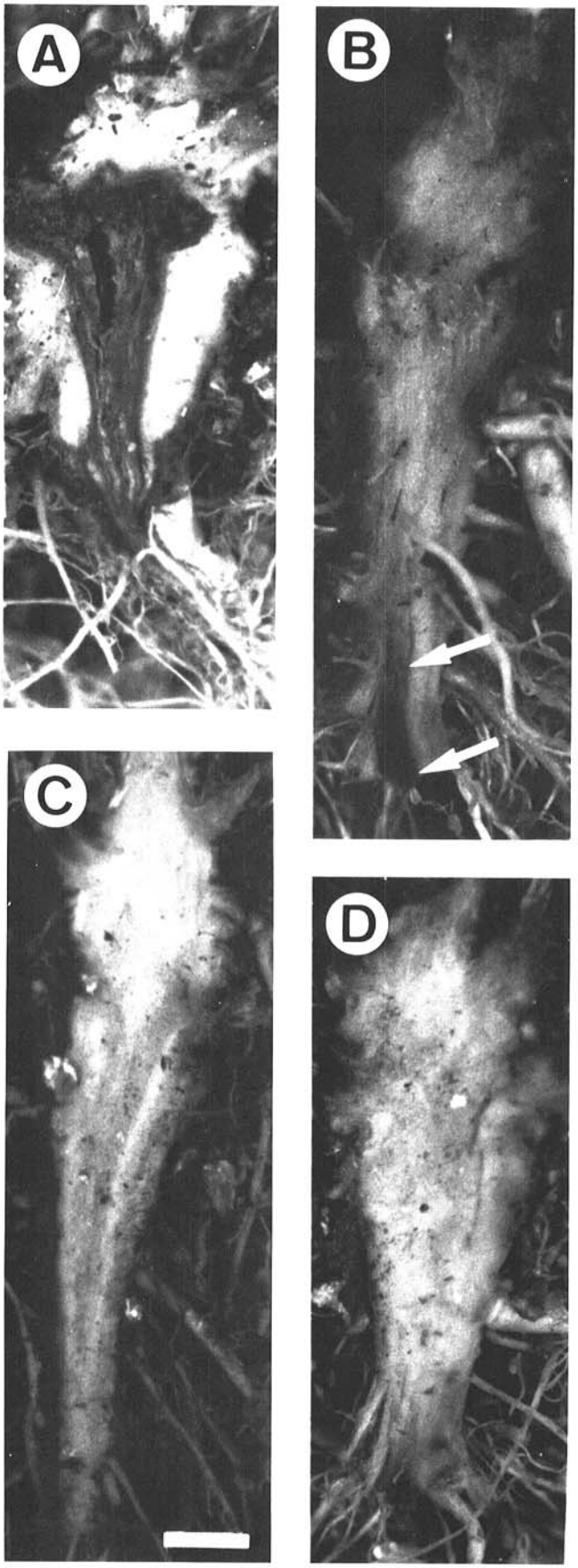

Fig. 1. Lesion characteristics of *Fusarium roseum* in the crown and root rot of clover. Panels represent longitudinally split taproots of infected and control plants. **A,** Characteristic lesion caused by isolate 5215; **B,** characteristic lesion caused by isolate 766; **C,** uncut and uninoculated control; and **D,** cut and uninoculated control. Arrows indicate the zone of greatest tissue discoloration caused by isolate 766. Bar = 1 cm.

28.8 ± 0.5% of water controls, respectively). These isolates are generally considered to be among the most pathogenic. Extracts from isolate 927 were the least phytotoxic (average seedling length was 51.1 ± 8.9% of water controls). Athough isolate 766 is considered to be only weakly pathogenic, it and the remaining isolates yielded intermediate phytotoxicity. There were generally no significant differences in extract phytotoxicities at 0.5× concentrations or greater. Overall, isolate toxigenicity did not correlate well with pathogenicity, which is in agreement with the observations of Stutz et al (14).

The results presented here for isolate 766 are markedly different from those obtained by other workers (14). Stutz et al (14) showed that isolate 766 had no biological activity as judged by electrolyte leakage, seedling elongation, and spore germination in a nutrient-poor culture filtrate of this isolate. Media composition and culture conditions can have a variety of marked effects on toxin production (12). Our observations suggest that isolate 766 may become toxigenic when provided with abundant nutrients in pure culture.

When crude extracts of filtrates from isolates 5215 and 766 were analyzed by TLC, both extracts were found to be composed primarily of two phytotoxic substances with R_f values of 0.28 and 0.38. There was essentially little or no significant difference between the yield of phytotoxic substances from the corresponding extract TCL regions of each isolate (*data not shown*).

Host specificity of crude filtrate extract from isolate 5215. Root elongation of all species tested was inhibited by the crude filtrate extract from isolate 5215, but some host specificity was observed. At essentially all concentrations of crude extract tested, nonhosts cucumber and timothy were the most resistant. At the 1× concentration root elongation of these plants was reduced by about only 50%. On the other hand, at the lowest concentrations (0.125× and 0.25×) root elongation of Florex and Arlington red clover were inhibited more than 50% by crude extracts. Lettuce, carrot, radish, tomato, and the two alfalfa varieties, Anchor and WL316, were all intermediate in their responses.

Phytotoxin purification and preliminary characterization. Phytotoxic components of pooled crude extracts of culture filtrates from isolate 5215 were subjected to relatively large-scale purification to obtain sufficient material for further chemical characterization studies. The majority of the phytotoxic activity was recovered in fraction II during silica gel column chromatography (Table 2). The greatest mass was also recovered in this fraction. Tests for fast and slow ninhydrin reactive substances suggest that column fractions and crude extracts contain both primary and secondary amino acids and that fraction II is enriched in secondary amino acids and contains a major phytotoxic component of the crude extract. The analyses would not allow estimation of the proportions of primary vs. secondary amino acids in these samples.

Thin-layer chromatography and seedling elongation bioassays revealed that column fraction II contained the two phytotoxic substances corresponding to those regions having TLC R_f values of 0.28 and 0.38 described earlier. Preparative TLC purification of these substances yielded crude crystalline and oily substances. Some structural information on these two compounds was afforded by NMR analysis. Proton NMR data of the crystalline

TABLE 1. Crown rot lesion lengths caused by different isolates of *Fusarium roseum*

Treatment	Mean lesion length (cm ± SE)
Isolate 5215	2.14 ± 0.18 a[z]
Isolate 814	1.30 ± 0.20 b
Isolate 959	1.21 ± 0.14 b
Isolate 927	1.18 ± 0.39 b
Isolate 766	0.83 ± 0.12 bc
Uninoculated cut control	0.99 ± 0.37 b
Uninoculated uncut control	0.21 ± 0.15 c

[z]Treatments with the same Duncan grouping are not significantly different at $P = 0.05$.

TABLE 2. Silica gel column fractionation of phytotoxicity of crude filtrate extract from *Fusarium roseum* isolate 5215[z]

Column fraction	Mass recovery (%)	Seedling length (% of water control) at relative concentration			
		0.5×	1×	2×	4×
I	1.7	94.5 ± 7.3 a	90.4 ± 17.8 a	90.0 ± 5.4 b	119.2 ± 6.7 a
II	32.7	33.5 ± 1.4 c	27.0 ± 2.1 b	22.6 ± 1.9 c	11.9 ± 2.3 e
III	27.1	68.7 ± 9.6 b	90.3 ± 7.8 a	84.2 ± 12.2 b	63.1 ± 0.6 d
IV	2.7	86.4 ± 7.1 ab	87.2 ± 4.2 a	84.0 ± 6.0 b	80.7 ± 5.1 cd
V	2.7	102.1 ± 7.2 a	109.7 ± 6.6 a	101.6 ± 5.0 ab	110.6 ± 4.6 ab
VI	10.1	107.9 ± 6.8 a	109.2 ± 5.9 a	109.9 ± 5.9 a	112.0 ± 5.6 ab
VII	14.1	100.7 ± 5.2 a	103.6 ± 8.8 a	97.4 ± 5.9 ab	100.8 ± 4.7 bc
VIII	4.4	92.6 ± 7.4 a	107.4 ± 3.1 a	95.6 ± 3.2 ab	106.8 ± 5.2 ab
Crude	100.0	26.6 ± 0.1 d	19.3 ± 1.3 b	5.3 ± 0.8 d	1.3 ± 0.5 f

[a]Data were arc sine-square root transformed before statistical analysis. Seedling lengths represent the means of three determinations consisting of the average length of 25 seedlings each. The average length of water control seedlings was 19.4 ± 1.5 mm. Data within a column followed by the same letter are not significantly different at $P = 0.05$ using Duncan's new multiple range test. Column fraction concentrations are expressed relative to the original culture filtrate. Total extract recovery was 95.45% of 547 mg of crude extract applied to column.

material in deuterated chloroform revealed a simple spectrum 4.15(m), 2.17(m), 1.07(s), and 0.93(s) ppm. These correspond in chemical shift and multiplicity to the alpha, beta, and methyl protons of the >CH-CH-$(CH_3)_2$ moiety of valine. This was confirmed by decoupling experiments. The absence of a singlet at about 3.0 ppm ruled out the presence of *N*-methyl groups in this sample. Proton NMR of the oily substance in deuterated acetone gave similar signals at 4.00 and 4.87 ppm, 2.35 and 2.52 ppm, and 0.87–1.17 ppm for the alpha, beta, and methyl protons of the >CH-CH-$(CH_3)_2$ moieties of both valine and isovaleric acid in addition to signals at 3.6, 7.3, and 7.25 ppm. The latter correspond to the methylene, *ortho* plus *meta*, and *para* protons, respectively, of a -CH_2-phenyl moiety. Valine, isovaleric acid, and benzyl moieties were estimated to be present in a ratio of 2:2:1, respectively. The singlet at 3.0 ppm clearly indicated the presence of an *N*-methyl moiety. Resonance characteristic for lactate moieties were not detected for either sample.

These preliminary analyses indicate that these phytotoxic substances are very similar but not identical to the cyclodepsipeptide enniatins, valinomycin antibiotics, and cyclodepsipeptide beauvericin produced by other fungi, including *F. roseum* (2–4,11). The substances described here are unique in that the enniatin B contains both *N*-methyl valine and isovaleric acid moieties, whereas they were not detected in the crystalline material. Valinomycin contains both valine and isovaleric acid moieties, which were detected in the oily substance. However, valinomycin also contains lactic acid residues, which were not detected in the substances descibed here. Lastly, while beauvericin does contain the aromatic -CH_2-phenyl and isovaleric acid moieties, it does not contain the *N*-methyl substitution or valine moieties of the oily substance. The precise roles, if any, of these phytotoxic substances in the root and crown rot disease of red clover remain to be determined.

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