(dd, 1H, J = 10.4, 2.3 Hz), 4.95 (m, 1H, J = 6.0 Hz), 5.24 (dt, 1H, J = 15.1, 7.5 Hz), 5.47 (bt, 1H), 5.50 (dd, 1H, J = 15.1, 6.6 Hz), 5.98 (d, 1H, J = 8.9 Hz); CIMS(CH_4) 471(M + 1), CIMS(NH_3) 488(M + 18); EIMS 427(M − 43)(10), 411(15), 367(37), 308(42), 133(80), 95(100), 55(95).

7: oil $[\alpha]_D$ − 3.1 (c 0.005, MeOH); UV(MeOH) λ_{max} 220 nm (ε 14000); IR 3300, 2930, 2860, 1715, 1650, 1542, 1452, 1222, 1150, 1105, 1048 cm^{-1} (NaCl plate); ^1H NMR (500 MHz, see table 1); ^{13}C NMR (75.5 MHz) 16.7 (q), 19.2 (q), 20.4 (q), 21.0 (t), 25.4 (t), 25.9 (t), 27.2 (t), 28.4 (t), 28.8 (t), 28.9 (t), 32.3 (t), 36.5 (t), 41.7 (2C, t), 43.0 (t), 44.7 (d), 50.4 (d), 63.7 (t), 64.8 (t), 68.8 (d), 70.3 (d), 71.1 (d), 74.8 (d), 117.4 (d), 129.4 (d), 134.3 (d), 157.1 (s), 166.9 (s), 171.9 (s), 173.6 (s); HRFABMS 567 (M + 1) 567.3610, $C_{30}H_{51}O_8N_2$ requires 567.3645, 257.1838, $C_{13}H_{25}O_3N_2$ requires 257.1865.

Acetylation of **7.** Compound **7** (3 mg) was acetylated as above. The acetate **8**, was isolated as an oil (3.5 mg). ^1H NMR (300 MHz, see table 1), EIMS 692(1), 632(1), 512(1), 257(15), 156(15), 115(46), 43(100).

Hydrolysis of **7.** **7** (2.0 mg) was dissolved in MeOH (1 ml), and 3 drops of 0.1 M KOH was added. The mixture was stirred for 24 h, the MeOH removed and the residue dissolved in water /EtOAc. The organic layer was removed, dried and the solvent removed to give an oil. This oil was acetylated (Py/Ac_2O) to give a product identical to **5** isolated from above. The water layer from above was acidified and extracted with EtOAc (3 ×). These organic extracts were treated with an ethereal solution of CH_2N_2 and the resulting oil subjected to HPLC on Partisil PXS 5/25 analytical column to give the methyl esters **9** (0.6 mg) and **10** (0.3 mg) as oils.

9: ^1H NMR (300 MHz) 1.00 (d, 3H, J = 7.3 Hz), 1.18 (d, 3H, J = 6.8 Hz), 2.04 (bs, 3H), 2.64 (db, 1H, J = 15.3 Hz), 3.42–3.55 (m, 3H), 3.69 (s, 3H), 3.70 (m, 1H), 3.80 (dd, 1H, J = 12.0, 3.0 Hz), 3.92 (bs, 1H), 5.42 (dd, 1H, J = 15.3, 7.4 Hz), 5.49 (dd, 1H, J = 15.3, 6.7 Hz), 5.70 (bs, 1H).

10: ^1H NMR (300 MHz) 0.97 (d, 3H, J = 7.0 Hz), 1.14 (d, 3H, J = 6.5 Hz), 1.37 (s, 3H), 2.65 (s, 2H), 3.50 (m, 2H), 3.64 (m, 2H), 3.66 (s, 3H), 3.82 (m, 1H), 4.11 (bs, 1H), 5.42 (m, 2H); EIMS 342(10), 324(22), 298(95), 211(65), 172(68), 110(95), 88(93), 43(100).

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1 Grayson, M., ed., Antibiotics, Chemotherapeutics, and Antibacterial Agents for Disease Control. John Wiley & Sons, New York 1982.
2 Lee, M. D., Dunne, T. S., Chang, C. C., Ellestad, G. A., Seigel, M. M., Morton, G. O., McGahren, W. J., and Borders, D. B., J. Am. chem. Soc. *109* (1987) 3466.
3 Golik, J., Dubay, G., Groenewold, G., Kawaguchi, H., Konishi, M., Krishnan, B., Ohkuma, H., Saitoh, K., and Doyle, T. W., J. Am. chem. Soc. *109* (1987) 3462.
4 Faulkner, D. J., Marine nat. Prod. Reports *51* (1988) 613.
5 Blade-Font, A., Tetrahedron Lett. *21* (1980) 2443.
6 Clayton, J. P., O'Hanlon, P. J., and Rogers, N. H., Tetrahedron Lett. *21* (1980) 881.

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Identification and phytotoxicity of 3-nitropropanoic acid produced in vitro by *Melanconis thelebola*

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Abstract. A phytotoxic metabolite was found to accumulate in culture of a strain of *Melanconis thelebola*, isolated from canker on red alder (*Alnus rubra* Bong.). The metabolite was identified by elemental analysis, ^1H- and ^{13}C-NMR and IR spectroscopy as 3-nitropropanoic acid.
Key words. Melanconis thelebola; phytotoxins; aliphatic carboxylic acid; 3-nitropropanoic acid.

Melanconis thelebola (Fr.) Sacc. is well known in Europe and North America as the causal agent of cankers on the trunk and branches of many alder species (*Alnus cordata, A. glutinosa, A. incana* and *A. rubra*)[1,2]. The fungus causes extensive dieback of the crown in both forest broadleafs and spontaneous broadleafs such as *A. rubra* Bong.; this species can be an important impediment to successful reforestation programmes, because of its rapid and unwanted growth. For this reason a study in British Columbia, Canada, has suggested that *M. thelebola* from cankerous *A. rubra* should be investigated for possible use in the biological control of broadleaf pests invading

forest stands[3]. Dorworth[4] likewise suggests the use of *M. thelebola* in forests as a mycoherbicide to check the spread of *A. rubra*.

On the other hand, a novel approach to weed biocontrol uses phytotoxins, rather than the fungus itself, as herbicides[5–8]. This paper reports on the purification and identification of a phytotoxin produced in culture by *M. thelebola*, and on its toxicity to tomato and alder.

Materials and methods

Production of culture filtrates. The *Melanconis thelebola* isolate used for in vitro toxin production was the Canadian isolate PCF012, provided by C. E. Dorworth, Pacific Forestry Centre, Victoria B.C., obtained from cankers on Red Alder (*Alnus rubra* Bong.) growing near Victoria. Ten 5-mm discs of the fungus mycelium from a 20-day-old culture on malt-extract-agar were transferred to 500-ml Erlenmayer flasks containing 200 ml of a modified Emerson broth (dextrose, 15 g; yeast extract, 6 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; distilled water, 1 l; pH 6) and incubated in shake culture (100 rpm) at 25 °C for 3 weeks. The cultures were then centrifuged (10,000 g × 15 min), and the supernatants filtered through a 0.45 µm Millipore membrane and adjusted to pH 5.5 with 1N NaOH. Part of the resulting filtrate was used immediately for the phytotoxicity assay, and part was stored at −30 °C for further processing.

Phytotoxic bioassay. The presence of toxin in the culture filtrate was routinely detected at different stages of the purification process by placing tomato cuttings in small vials containing filtrate for 24 h at 26 °C under continuous light, and then transferring the cuttings to distilled water for a further 24 h[9,10]. Toxicity of the purified toxin was also tested on alder leaves using the pin-prick method[11]. The toxin (1 mg) was dissolved in a small amount of methanol, taken up in distilled water, the pH adjusted to 5.5 with NaOH 1N, and diluted 1-, 4- and 16-fold. 10-µl drops were then applied onto the lower side of leaves, which were then superficially scratched 24 times through the drop with a dissecting needle. Symptoms were observed 24 h after toxin application.

Phytotoxin extraction and purification. The culture filtrate (3.0 l) was lyophilized and the residue dissolved in 0.6 l distilled H_2O (1/5 of initial filtrate volume). The solution was adjusted to pH 2.5 with 99% formic acid and extracted with ethyl acetate (4 × 400 ml). The combined organic extracts were dried with sodium sulphate (Na_2SO_4), filtered, and evaporated under low pressure. An aliquot of the crude residue, dissolved in methanol, was analyzed by TLC on silica gel (Merck, Kieselgel 60 F_{254}, 0.25 mm) plates; eluent: *n*-butanol-acetic acid-water (60 : 15 : 25, v/v/v). The chromatograms were developed with iodine vapor and/or by spraying with 0.5% ninhydrin in acetone, followed by heating at 110 °C for 10 min. The residue (1.36 g) left from the organic extracts was fractionated on a 2.5 × 70 cm Sephadex LH-20 column (Pharmacia); eluent: chloroform-ethyl acetate-methanol (2 : 2 : 1, v/v/v), elution rate: 3 ml/min, fraction volume: 3 ml. The fractions collected were analyzed by TLC as above; groups of homogeneous fractions were combined and assayed for toxicity on tomate cuttings. The residue (324 mg) left from the groups of combined toxic fractions was purified in two separate portions on a 2.0 × 100 cm silic acid (100 mesh, Mallinckrodt) column; eluent: chloroform-*iso*-propanol (9 : 1, v/v), elution rate: 60 ml/h, fraction volume: 1.5 ml. The fractions collected were analyzed by TLC as above. The fractions containing the main component (colored yellow-pink after spraying the plates with the ninhydrin solution) were combined and evaporated under reduced pressure. The homogeneous oily residue (197 mg), which was toxic on tomato cuttings, was crystallized as white needles from chloroform at − 20 °C (116 mg, 38.6 mg/l).

Toxin identification. The melting points (uncorrected) were measured on a Kofler block, while elemental analysis was performed on a 740 Perkin Elmer instrument. The natural compound was identified by ^{1}H- and ^{13}C-NMR, recording the spectra in CD_3OD at 270 and 67.92 MHz, respectively, on a Bruker, A.C. 270 spectrometer, using the same solvent as for the internal standard. The IR spectra were recorded in neat on a Perkin-Elmer IR-FT 1720-X spectrometer, while the UV spectra were measured in methanol on a Perkin-Elmer, 550 S spectrophotometer. EI-MS and CI-MS were recorded at 70 and 300 eV, respectively, on a Kratos MS-80 spectrometer, using *iso*-butane as a reagent gas for the CI-MS.

Chemical derivatization of the toxin. The acid toxin (32.8 mg), dissolved in 4 ml methanol, was converted into the corresponding methyl ester by reaction with ethereal diazomethane. The crude product was purified on a silica gel column (eluent: chloroform-*iso*-propanol, 97 : 3, v/v), yielding a homogeneous oily compound (27.9 mg) which was identified by ^{1}H-NMR (recorded in $CDCl_3$) and by IR and UV spectroscopy performed as above.

Results

Toxic activity. A toxic effect on tomato cuttings was noted for 21-day-old culture filtrates of *M. thelebola*. It

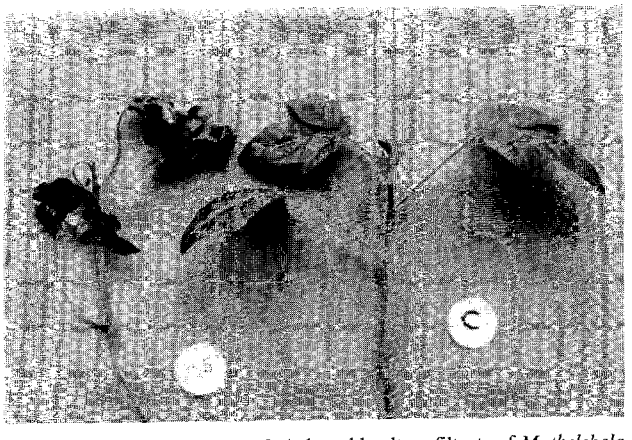


Figure 1. Toxic effects (left) of 21-day-old culture filtrate of *M. thelebola* on tomato cuttings; control (right): uninoculated growth medium.

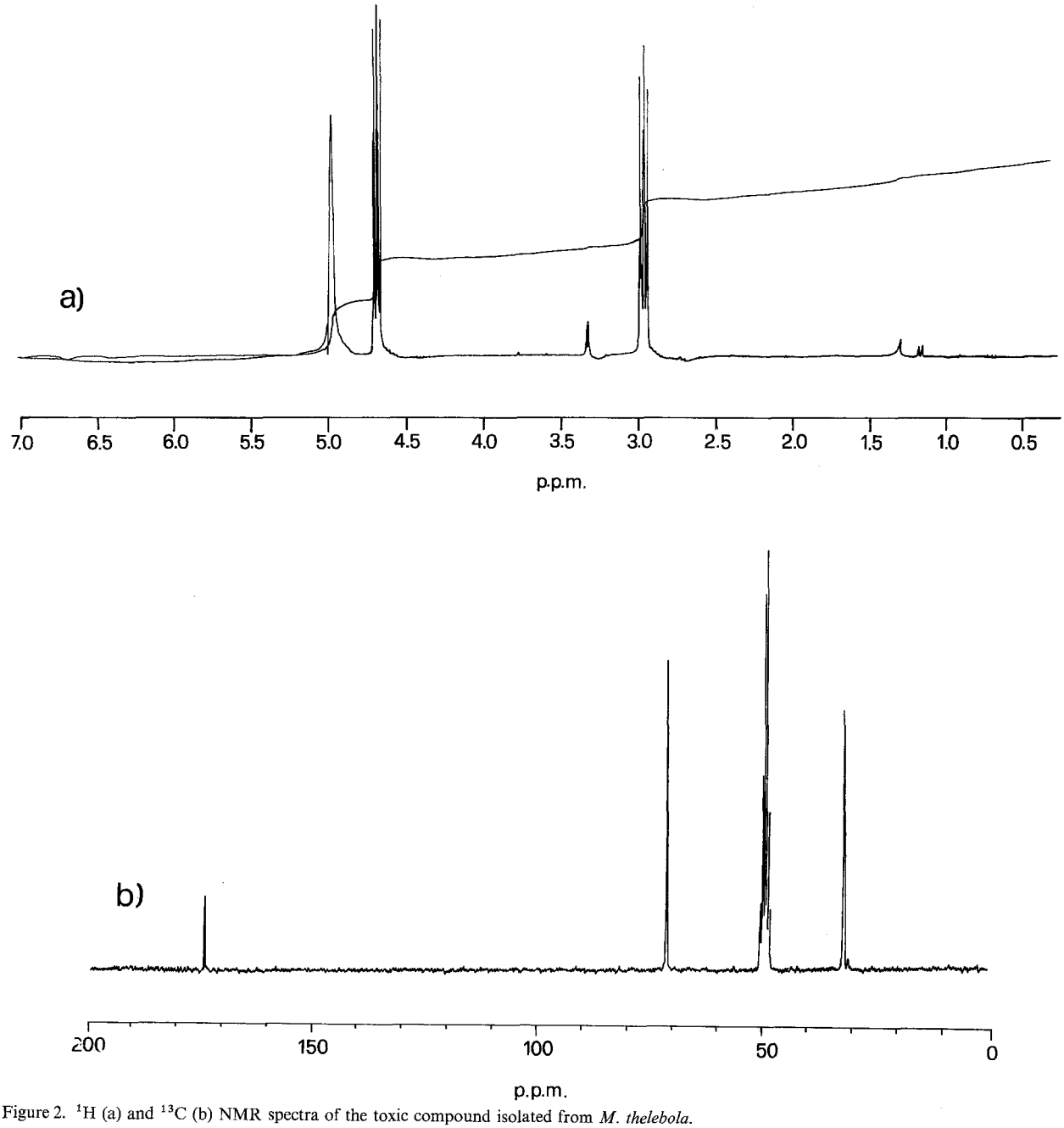
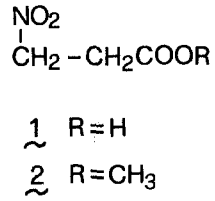


Figure 2. ^{1}H (a) and ^{13}C (b) NMR spectra of the toxic compound isolated from *M. thelebola*.

caused a collapse of the petioles accompanied by complete wilting of the leaves (fig. 1). Flabbiness of the petioles appeared within 3–4 h and was complete after 20 h. Controls using uninoculated medium gave no reaction in this test. Culture filtrates diluted one- and fourfold induced only slight petiole collapse and small necrotic spots on the leaf lamina. Petiole collapse was also induced by the purified toxin at 3 mg/ml (2.5×10^{-2} M). The minimum toxin concentration which induced visible effects on the tomato cuttings was 1.13 µmoles/g f.w. When 10-µl droplets of the purified toxin at a concentration of 2.5×10^{-2} M were applied to alder leaves, tan colored lesions were produced at the area of application. An authentic sample of 3-nitropropionic acid (Aldrich, Chemical Co., Milwaukee, U.S.A.) assayed on tomato and alder leaves induced the same phytotoxic effects caused by the natural toxin.

Toxin identification. The phytotoxic metabolite produced in vitro by *M. thelebola* and shown by elemental analysis to have a molecular formula of $C_3H_5NO_4$ was identified as 3-nitropropanoic acid by spectroscopic analysis. The ^{1}H-NMR spectrum (fig. 2a) showed two coupled triplets at δ 4.68 (2H, $J = 6.2$ Hz) and 2.95 (2H, $J = 6.2$ Hz) assigned to two CH_2 groups, bonded to a NO_2 and a



COOH group respectively [12]. The same structural feature was observed with the ^{13}C-NMR data (fig. 2b): the signals of the two methylene groups resonated at δ 70.1 and 31.7, while the carboxylic group appeared as a singlet at δ 173.5 [13]. The presence of the carboxylic and nitro group was confirmed by the appearance of the typical bands in the IR spectrum at 3018 (broad) and 1724 and at 1558 cm^{-1}, while the UV spectrum showed the weak absorption at 270 nm ($\varepsilon = 20.6$) [12, 14].

Both the EI and the CI mass spectra gave the pseudomolecular ion at m/z 120 $[MH]^+$ and the characteristic fragmentation peaks at m/z 103 $[MH-OH]^+$, 102 $[M-OH]^+$, 74 $[MH-NO_2]^+$, 73 $[M-NO_2]^+$ and 55 $[M-NO_2-H_2O]^+$ (base peak) [12].

The structure **1** assigned to the toxic metabolite was confirmed by preparing the corresponding methyl ester (**2**) which differed from it only for the presence of the methoxy group at δ 3.74 and for the lacking of OH absorptions in the ^{1}H and IR spectrum, respectively.

The natural toxin and an authentic sample of 3-nitropropionic acid had the same chromatographic behavior and melted at the same temperature (65–67 °C) alone or in mixture [15]. Finally, under identical experimental conditions, the toxin (**1**) also had the same IR, UV, ^{1}H- and ^{13}C-NMR and MS spectra as 3-nitropropionic acid, and the chemical and physical properties of **1** were very close to those reported in the literature for 3-nitropropionic acid [16].

Discussion

The toxic metabolite produced in culture from *M. thelebola* was isolated and identified as 3-nitropropanoic acid (**1**), also known as β-nitropropionic acid or hiptagenic acid, a toxin [16] naturally produced by some fungi including *Aspergillus* [16, 17] and *Penicillium* [16, 18] species. β-Nitropropionic acid is also a metabolite of some families of higher plants that are important as forage [15, 19], where it occurs either as a free compound or as a conjugate obtained by esterification with glucose [19, 20, 21].

β-Nitropropionic acid is toxic to animals [15, 16, 19] and to insects [21], but its toxicity to plants is reported here for the first time. This is also the first time that **1** is identified as a metabolite of *M. thelebola*. Phytotoxic, low molecular weight organic acids are produced by fungi pathogenic to plants. Examples are oxalic acid [22], fumaric acid [23] and 3-methylthiopropionic acid [24]. The latter compound, isolated from in vitro culture of *Xanthomonas campestris* pv. *manihotis*, the causal agent of cassava blight, was also found in the infected leaves of the same plant, suggesting it could have a role in the disease caused by the bacterium [24].

3-Methylthiopropionic acid is structurally very close to **1**, and since the two compounds differ only in the terminal group bonded at the C-3 of the propanoic acid chain, it may be hypothesized that the toxicity is associated with the aliphatic acid chain, and that the substituent group (NO_2 or SCH_3) at C-3 is relatively less important in causing the toxic effects.

Further studies are needed to determine whether production of 3-nitropropanoic acid by *M. thelebola* has a role in the disease process and if and how the toxin may be used as a forest herbicide. Preliminary observations indicated that *A. rubra* seed germination is inhibited to some extent by 3-nitropropanoic acid.

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* Author for correspondence
1 Saccardo, P. A., Sylloge fungorum *1* (1882) 605.
2 Sutton, D. C., The Coleomycetes. Fungi imperfecti with pycnidia acervuli and stomata, p. 635. CAB McLohose, R. & Co. Ltd., University of Glasgow 1980.
3 Tebeest, D. O., and Tempton, G. E., Plant Dis. *69* (1985) 6.
4 Dorworth, C. E., Mycoherbicides for forest weed control. The PCF enhancement process. Proceeding of the Alternative to the Chemical Control of Weeds Conference, Rotoa, July 25–27 1989. Ed. C. Basset. Forest Research Institute, New Zealand.
5 Cutler, H. G., The Science of Allelopathy, p. 147. Eds A. R. Putnam and C. S. Tang. Wiley-Interscience, New York 1986.
6 Duke, S. O., Rev. Weed Sci. *2* (1986) 15.
7 Strobel, G. A., Sugawara, F., and Clardy, J., Allochemicals: Role in Agriculture and Forest, ACS Symposium Series, n. 300, p. 516. Ed G. R. Waller. American Chemical Society, Washington, D.C. 1987.
8 Kenfield, D., Bunkers, G., Strobel, G. A., and Sugawara, F., Fungal phytotoxins potential new herbicides, in: Phytotoxins and Plant Pathogenesis, p. 319. Eds A. Graniti, D. R. Durbin and A. Ballio. Springer-Verlag, Berlin 1989.
9 Graniti, A., Phytopath. medit. *22* (1964) 120.
10 Bottalico, A., Phytopath. medit. *11* (1972) 1.
11 Mitchell, R. E., and Bieleski, R. L., Pl. Physiol. *60* (1977) 723.
12 Silverstein, R. M., Bassler, G. C., and Morrill, T. C., Spectrometric Identification of Organic Compounds, pp. 27, 99, 211, 239. J. Wiley & Sons Inc., New York 1977.
13 Wherly, F. W., and Wirthlin, T., Interpretation of Carbon-13 NMR Spectra, p. 23. Heyden, London 1976.
14 Nakanishi, K., and Solomon, P. H., Infrared Absorption Spectroscopy, pp. 25 and 45. Holden Day Inc., Oakland 1977.
15 Morris, M. P., Pagan, C., and Warmke, M. E., Science *119* (1954) 322.
16 Cole, R. J., and Cox, R. H., Handbook of Toxic Fungal Metabolites, p. 755. Academic Press, New York 1981.
17 Bush, M. T., Touster, O., and Brockman, J. E., J. biol. Chem. *188* (1951) 685.
18 Raistrick, H., and Stossl, A., Biochem. J. *68* (1958) 647.
19 Williams, M. C., Can. J. Bot. *60* (1982) 1956.
20 Majak, W., and Bose, R. J., Phytochemistry *15* (1976) 415.
21 Byers, R. A., Gustine, D. L., and Moyer, B. G., Environ. Entomol. *6* (1977) 229.
22 Noyes, R. D., and Hancock, J. G., Physiol. Pl. Path. *18* (1981) 123.
23 Mirocha, C. J., De Vay, J. E., and Wilson, E. E., Phytopathology *51* (1961) 851.
24 Perreaux, D., Maraite, H., and Meyer, J. A., Physiol. Pl. Path. *20* (1982) 313.

0014-4754/92/11-12/1169-04$1.50 + 0.20/0
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