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Metabolites Inhibiting Germination of *Orobanche ramosa* Seeds Produced by *Myrothecium verrucaria* and *Fusarium compactum*

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Myrothecium verrucaria and *Fusarium compactum* were isolated from diseased *Orobanche ramosa* plants collected in southern Italy to find potential biocontrol agents of this parasitic weed. Both fungi grown in liquid culture produced metabolites that inhibited the germination of *O. ramosa* seeds at 1−10 μM. Eight metabolites were isolated from *M. verrucaria* culture extracts. The main metabolite was identified as verrucarin E, a disubstituted pyrrole not belonging to the trichothecene group. Seven compounds were identified by spectroscopic methods as macrocyclic trichothecenes, namely, verrucarins A, B, M, and L acetate, roridin A, isotrichoverrin B, and trichoverrol B. The main metabolite produced by *F. compactum* was neosoloaniol monoacetate, a trichothecene. All the trichothecenes proved to be potent inhibitors of *O. ramosa* seed germination and possess strong zootoxic activity when assayed on *Artemia salina* brine shrimps. Verrucarin E is inactive on both seed germination and zootoxic assay.

KEYWORDS: ***Orobanche ramosa*; *Myrothecium verrucaria*; *Fusarium compactum*; phytotoxins; trichothecenes; seed germination inhibitors**

INTRODUCTION

Orobanche ramosa L. (broomrape) is a widespread parasitic weed of many Solanaceae species, such as tobacco or tomato, and attaches to many other species, including ornamentals and weeds. It is distributed mainly in the Mediterranean area, central Europe, northern Africa, and the Middle East (*1*). By interfering with water and mineral intake and by affecting photosynthate partitioning, it is responsible for both qualitative and quantitative damage to crops. Difficulties in broomrape control are in part due to the large amount of seeds produced that can remain viable for many years. They germinate only by stimulation with host root exudates, and produce a germ tube that, if it attaches to the host root, develops a haustorium penetrating the root and forms a tubercle. This is followed by the most damaging phase, with the parasitic withdrawal of water, nutrients, and photosynthates from the host. Due to the long underground phase, plant emergence occurs only when most of the damage has already been produced.

Traditional control methods have been tried on different crops, but none have proved to be effective. *Orobanche* sp. cannot usually be managed by persistent selective herbicides, since herbicides are not able to differentiate between the crop and the parasite, except on herbicide-resistant transgenic crops (*2*). Multiple applications of low rates of crop-degraded herbicides can provide a modicum of control and may be more useful when integrated with other methods (*3*). Seed eradication by solarization or soil fumigation with methyl bromide and ethylene dibromide are effective but expensive, and fumigation is mostly banned due to environmental risks (*4*). Furthermore, as these weeds attach to crop roots, they cannot be controlled mechanically, except by removing their flower stalks to reduce seed accumulation and dispersal. No pathogenic organisms have been yet developed as a mycoherbicide for *Orobanche* biocontrol, although many promising agents have been isolated (*5*).

Considering that stimulated seed germination is a key phase of the parasitic plant life cycle, the search for natural compounds able to inhibit the germination appears to be an attractive and environmentally friendly approach. Some toxins produced by fungi of the genus *Fusarium* proved to be able to inhibit germination of *O. ramosa* seeds, and their practical use in integrated strategies of parasitic plant management has been proposed (*6*). Recently, many fungi were isolated from diseased *O. ramosa* plants during extensive field surveys carried out within a national project, and some of them proved to be promising potential mycoherbicides for biological control of broomrapes (*7*). Fifty-three isolates tested for virulence were also grown in vitro on both liquid and solid media with the main aim to find new metabolites having the ability to inhibit the induced germination of *O. ramosa* seeds (*8*). All the extracts from the liquid culture were assayed for the ability to inhibit

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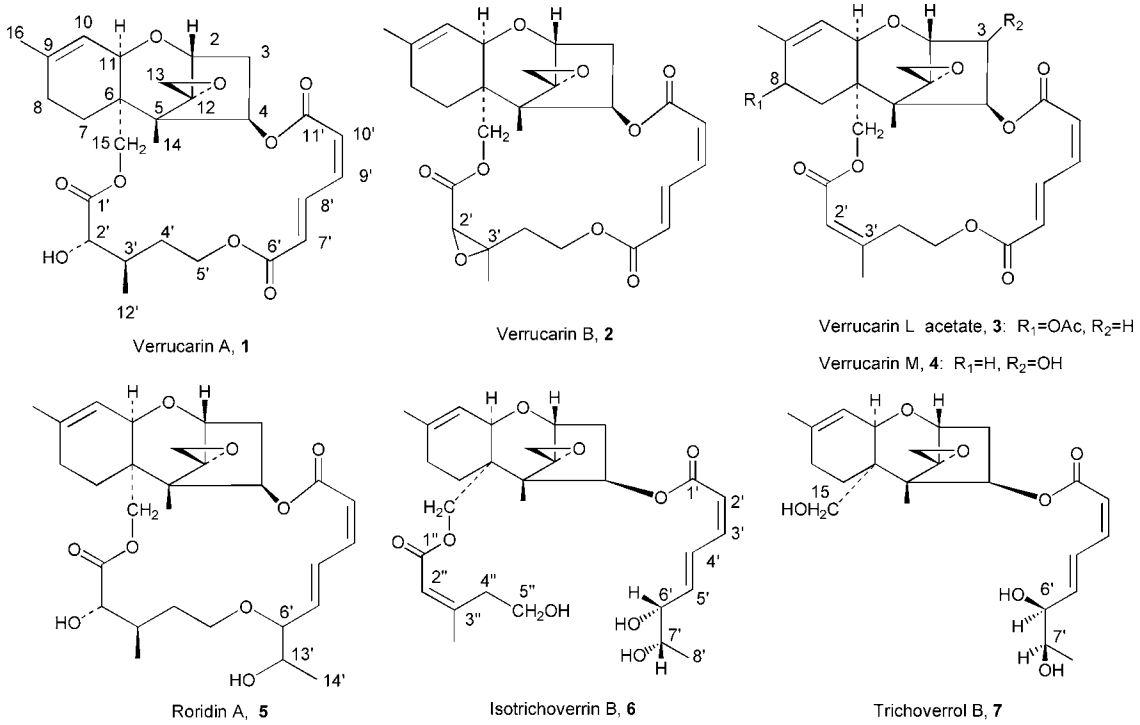

Figure 1. Chemical structure of verrucarins A, B, L acetate, and M, roridin A, isotrichoverrin B, and trichoverrol B (**1**, **2**, **3**, **4**, **5**, **6**, and **7**, respectively).

seed germination, and most of them proved to be ineffective or only slightly active. Only the extracts produced by five strains (*Fusarium compactum* sensu Gordon, *Myrothecium verrucaria* (Albertini et Schweinitz) Ditmar:Fries, *Alternaria* sp., *Fusarium equiseti* (Corda) Sacc., and *Fusarium* sp.) were highly effective, causing the total or nearly complete inhibition of germination, and were further considered as sources of new natural compounds (*8*). In this study we have investigated the production, the chemical purification, and the chemical and biological characterization of the metabolites produced by *M. verrucaria* and *F. compactum*.

MATERIALS AND METHODS

Fungus. *M. verrucaria* and *F. compactum* were isolated during extensive surveys in fields heavily infested by *O. ramosa* in the South of Italy (*7*). They are stored in the collection of the Istituto di Scienze delle Produzioni Alimentari, CNR, Bari, Italy, as ITEM 6168 and ITEM 6160, respectively.

General Experimental Procedures. Optical rotation was measured in $CHCl_3$ solution on a Jasco (Tokyo, Japan) P-1010 digital polarimeter. Infrared (IR) spectra were recorded as neat on a Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer. UV spectra were taken in EtOH solution on a Lambda 25 UV−vis spectrophotometer. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 500, 400, or 300 MHz and at 125, 100, or 75 MHz, respectively, in $CDCl_3$, on Bruker (Karlsrhe, Germany) spectrometers. The same solvent was used as the internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectroscopy (*9*). DEPT−correlated spectroscopy (COSY) experiments (*9*) were performed using Bruker microprograms. Electron ionization (EI) spectra were taken at 70 eV on a Fisons Trio-2000 spectrometer. Electrospray ionization (ESI) mass spectra were recorded on a Perkin-Elmer (Norwalk, CT) API 100 LC−MS instrument with a probe voltage of 5300 V and a declustering potential of 50 V. Analytical and preparative TLC were performed on Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively, silica gel plates (Merck, Darmstadt, Germany) or KC18 F_{254}, 0.20 mm, reversed-phase plates (Whatman, Clifton, NY); the spots were visualized by exposure to UV light at nanomolar concentration and/or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110 °C for 10 min. For column chromatography (CC) Kieselgel 60, 0.063−0.20 mm, silica gel (Merck) was used.

Single-Crystal X-ray Analysis of Verrucarin E (8). A colorless crystal (prism, 0.39 × 0.10 × 0.03 mm^3) was obtained from C_6H_6 solution (slow evaporation). Data were acquired on a Nonius Mach3 (Delft, Holland) single-crystal diffractometer (graphite-monochromated Mo Kα radiation); the structure was solved and refined by standard procedures using the Shelx-97 package. The crystal structure found was identical to that previously reported (*10*).

Production, Extraction, and Purification of *M. verrucaria* Phytotoxins. A conidial suspension (1 mL containing approximately 10^6 conidia) was added to 1 L Roux bottles containing 200 mL of the mineral medium M1-D (*11*) for the production of toxic metabolites. The cultures were incubated under static conditions at 25 °C in the dark for 4 weeks, then filtered first through cheesecloth and then on Whatman no. 4 filter papers at reduced pressure, and lyophilized for the successive purification steps.

The lyophilized material obtained from the culture filtrates (1.5 L) was dissolved in 250 mL of distilled water (final pH 4.2) and extracted with ethyl acetate (3 × 250 mL). The organic extracts were combined, dried with Na_2SO_4, filtered, and evaporated under reduced pressure. The brown oil residue (510 mg) proved to be highly active in inhibiting the germination of *O. ramosa* seeds (100% inhibition), and was fractionated by CC eluted with $CHCl_3$−*i*-PrOH (19:1, v/v), yielding seven groups of homogeneous fractions, which were all assayed on *O. ramosa* seeds. Only the first four fractions proved to be highly phytotoxic (80−100% inhibition). TLC analysis [$CHCl_3$−*i*-PrOH (19:5, v/v) and EtOAC−*n*-hexane (1.5:1, v/v)] of their residues showed the presence of several metabolites. The residue of the first fraction (10.6 mg) was purified by preparative TLC [$CHCl_3$−*i*-PrOH (19:5, v/v)], affording two main bands, the first of which (2.4 mg) was further purified by preparative TLC [eluent EtOAC−*n*-hexane (1.5:1, v/v)], yielding verrucarin L acetate (**3**; **Figure 1**) as a white amorphous solid (R_f 0.60, 1.4 mg, 0.9 mg/L). The residue (2.5 mg) of the other band was purified by reversed-phase preparative TLC (eluent C) to give verrucarin A (**1**; **Figure 1**) as a white amorphous solid (R_f 0.60, 1.2 mg, 0.8 mg/L). The residue of the second active fraction (22.6 mg) of the column, containing two main metabolites, was further purified by two successive preparative TLC steps [eluent $CHCl_3$−*i*-PrOH (19:1, v/v) and eluent petroleum−Me_2CO (1.5:1, v/v)], yielding verrucarin B and verrucarin M (**2** and **4**; **Figure 1**), both as amorphous solids [R_f

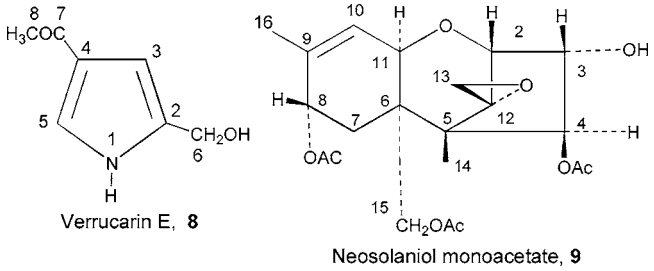

Figure 2. Chemical structure of verrucarin E (**8**) and neosolaniol monacetate (**9**).

0.43, eluent petroleum−Me_2CO (1.5:1, v/v), 0.6 mg, 0.4 mg/L, and R_f 0.50, eluent petroleum−Me_2CO (1.5:1, v/v), 0.8 mg, 0.5 mg/L, respectively]. The residue of the third active residue (45.0 mg) of the column, containing one main metabolite, was purified by two preparative TLC steps [eluent EtOAc−*n*-hexane (4:1, v/v) and reversed-phase eluent EtOH−H_2O (1:1, v/v)], yielding roridin A (**5**; **Figure 1**) as an amorphous solid (R_f 0.37, eluent E, 13.0 mg, 8.6 mg/L). The purification of the residue of the fourth active fraction (37.0 mg) of the same column, carried out by preparative TLC [silica gel eluent $CHCl_3$−*i*-PrOH (19:1, v/v)] yielded two main bands (9.0 and 6.5 mg): the first (9.0 mg) was further purified by preparative TLC [eluent EtOH−H_2O (1:1, v/v)], yielding isotrichoverrin B (**6**; **Figure 1**) as an amorphous solid (R_f 0.46, eluent EtOH−H_2O (1:1, v/v), 2.5 mg, 1.6 mg/L); the second band (6.5 mg), purified using the same conditions, gave trichoverrol B (**7**; **Figure 1**) always as an amorphous solid (R_f 0.60, eluent EtOH−H_2O (1:1, v/v), 2.0 mg, 1.3 mg/L). The residues of the fifth to seventh inactive fractions of the column (60.4, 86.1, and 64.3 mg, respectively), containing a metabolite in larger amounts, were combined and further purified by preparative TLC [eluent EtOH−H_2O (1:1, v/v)], yielding verrucarin E (**8**; **Figure 2**), as a white crystalline solid (R_f 0.25, eluent B, 100.0 mg, 66.6 mg/L) which was recrystallized as white needles by $CHCl_3$.

Verrucarin A (1). $[\alpha]_D$, IR, UV, ^{13}C NMR, and EI-MS data are very similar to those reported in the literature (*12*). ^1H NMR data previously reported (*12*) were integrated with the following signals: δ = 1.87 (2H, m, H-4′), 1.94 (2H, m, H-7), 2.02 (2H, m, H-8), 2.25 (1H, m, 3A), 2.46 (1H, m, 3B), 3.11 (1H, d, $J_{13A,13B}$ = 4.0 Hz, H-13B), 3.56 (1H, d, $J_{2',3'}$ = 3.9 Hz, H-3′), 3.86 (1H, d, $J_{10,11}$ = 4.4 Hz, H-11), 3.99 (1H, m, H-2), 4.22 (1H, d, $J_{15A,15B}$ = 12.1 Hz, H-15A), 4.52 (2H, m, H-5′), 4.80 (1H, d, $J_{15A,15B}$ = 12.1 Hz, H-15B). ESI-MS: *m*/*z* 525 $[M + Na]^+$.

Verrucarin B (2). IR and UV data are very similar to those reported in the literature (*12*). ^1H NMR data previously reported (*12*) were integrated with the following signals: δ = 1.90 (2H, m, H-7), 2.02 (2H, m, H-8), 2.34 (1H, m, 3B), 2.37 (2H, m, H-4′), 2.51 (1H, m, 3A), 2.86 (1H, d, $J_{13A,13B}$ = 4.0 Hz, H-13A), 3.45 (1H, d, $J_{13A,13B}$ = 4.0 Hz, H-13B), 3.62 (1H, d, $J_{2,3}$ = 3.7 Hz, H-2), 3.81 (1H, d, $J_{10,11}$ = 5.2 Hz, H-11), 4.32 (1H, m, 15A), 4.50 (1H, m, H-15B), 4.46 (2H, m, H-5′), 5.43 (1H, d, $J_{10,11}$ = 5.2 Hz, H-10). EI-MS: *m*/*z* (rel intens) 443 $[M - C_3H_7O]^+$ (2), 248 $[M - C_{15}H_{20}O_3]^+$ (12), 247 $[M - C_{13}H_{17}O_5]^+$ (35). ESI-MS: *m*/*z* 501 $[M + H]^+$, 523 $[M + Na]^+$, 539 $[M + K]^+$.

Verrucarin L Acetate (3). $[\alpha]_D$ and UV data are very similar to those reported in the literature (*13*). IR: ν_{max} (cm^{-1}) 1715 (ester C═O), 1270 (ester COO), 1078 (epoxide ring). ^1H NMR data previously reported (*13*) were integrated with the following signals: δ = 2.31 (1H, m, 3A), 2.43 (1H, m, 3B), 2.23 (2H, m, H-7), 2.49 (2H, m, H-4′), 3.79 (1H, d, $J_{2,3}$ = 5.5 Hz, H-2), 4.14 (2H, m, H-5′), 5.21 (1H, br s, H-8), 5.76 (1H, s, H-2′), 5.93 (1H, m, H-4). EI-MS: *m*/*z* (rel intens) 500 $[M - C_2H_2O]^+$ (5), 371 $[M - C_7H_7O_5]^+$ (7), 245 $[M - C_{14}H_{17}O_7]^+$ (35). ESI-MS (+): *m*/*z* 565 $[M + Na]^+$, 581 $[M + K]^+$.

Verrucarin M (4). $[\alpha]^{25}_D$ +33.4 (*c* 0.1). IR, UV, and ^1H NMR data are very similar to those reported in the literature (*14*). EI-MS: *m*/*z* (rel intens) 388 $[M - C_6H_9O_2]^+$ (32), 289 $[M - C_{10}H_{12}O_5]^+$ (5), 248 $[M - C_{12}H_{13}O_6]^+$ (12). ESI-MS (+): *m*/*z* 502 $[M + H]^+$, 524 $[M + Na]^+$.

Roridin A (5). $[\alpha]_D$, IR, UV, ^{13}C NMR, and EI-MS data are very similar to those reported in the literature (*12*). ^1H NMR data previously reported (*12*) were integrated with the following signals: δ = 1.12 (3H, d, $J_{3',12'}$ = 6.6, H-12′), 1.88 (2H, m, H-4′), 1.91 (2H, m, H-7), 2.02 (2H, m, H-8), 2.20 (1H, m, 3A), 2.45 (1H, m, 3B), 2.49 (1H, m, H-3′), 2.80 (1H, d, $J_{13A,13B}$ = 4.0 Hz, H-13A), 3.11 (1H, d, $J_{13A,13B}$ = 4.0 Hz, 13B), 3.50 (2H, m, H-5′), 3.55 (1H, m, H-6′), 3.60 (1H, m, H-2), 3.65 (1H, m, H-13′), 3.84 (1H, d, $J_{10,11}$ = 4.9 Hz, H-11). ESI-MS (+): *m*/*z* 533 $[M + H]^+$.

Isotrichoverrin B (6). $[\alpha]_D$ and ^1H NMR data are very similar to those reported in the literature (*15*). IR: ν_{max} (cm^{-1}) 3409 (OH), 1709 (ester C═O), 1074 (epoxide ring). UV: λ_{max} (log ϵ) 262 (4.42) nm. ESI-MS: *m*/*z* 533 $[M + H]^+$, 555 $[M + Na]^+$, 571 $[M + K]^+$.

Trichoverrol B (7). $[\alpha]_D$, UV, and ^{13}C NMR data are very similar to those reported in the literature (*13*). IR: ν_{max} (cm^{-1}) 3458 (OH), 1697 (ester C═O), 1076 (epoxide ring). ^1H NMR data previously reported (*13*) were integrated with the following signals: δ = 1.54 (1H, m, H-7B), 2.08 (3H, m, H-7A, H_2-8), 3.65 (1H, m, H-2) 3.68 (1H, d, $J_{15A,15B}$ = 12.7 Hz, H-15B), 3.82 (1H, d, $J_{15A,15B}$ = 12.7 Hz, H-15A), 3.87 (1H, d, $J_{10,11}$ = 5.1 Hz, H-11), 3.94 (1H, m, H-7′), 6.15 (1H, dd, $J_{4',5'}$ = 15.4, $J_{5',6'}$ = 5.6 Hz, H-5′). EI-MS: *m*/*z* (rel intens) 420 $[M]^+$ (1), 402 $[M - H_2O]^+$ (9), 388 $[M - MeOH]^+$ (25), 370 $[M - MeOH - H_2O]^+$ (11). ESI-MS: *m*/*z* 443 $[M + Na]^+$, 459 $[M + K]^+$.

Verrucarin E (8). IR, UV, and ^1H NMR data are very similar to those reported in the literature (*16*). ^{13}C NMR: δ = 124.6 (s, C-2), 118.4 (d, C-3), 124.2 (s, C-4), 127.5 (d, C-5), 57.4 (t, C-6), 196.4 (s, C-7), 27.1 (q, C-8). EI-MS: *m*/*z* (rel intens) 139 $[M]^+$ (100), 124 $[M - Me]^+$ (97), 108 $[M - CH_2OH]^+$ (65), 93 $[M - CH_3 - CH_2OH]^+$ (77).

Production, Extraction, and Purification of *F. compactum* Phytotoxins. The strain of *F. compactum* was grown as described for *M. verrucaria* for the production of toxic metabolite.

The lyophilized culture filtrates of *F. compactum* (11 L) were dissolved in distilled water (1/10 of the original volume, final pH 4) and extracted with ethyl acetate (3 × 1000 mL). The organic extracts were combined, dried with Na_2SO_4, filtered, and evaporated under reduced pressure. The brown oil residue (1.137 g), having a high phytotoxicity when assayed on *O. ramosa* seeds (100% inhibition of germination), was fractionated by CC eluted with $CHCl_3$−*i*-PrOH (9:1, v/v) to yield eight groups of homogeneous fractions. Only the second fraction proved to be highly phytotoxic (100% inhibition) when assayed on *O. ramosa* seeds. The residue of this fraction (40.8 mg), containing the main metabolite having R_f 0.25 by TLC [eluent petroleum−Me_2CO (2.3:1, v/v)], was further purified in the same conditions, producing neosolaniol monoacetate (**9**; **Figure 2**) as a homogeneous oily compound (8.0 mg, 0.7 mg/L).

Neosolaniol Monoacetate (9). $[\alpha]^{25}_D$ +77 (*c* 0.1). IR, UV, and ^1H NMR data are very similar to those reported in ref *17*. EI-MS: *m*/*z* (rel intens) 382 $[M - CH_2CO]^+$ (5), 364 $[M - AcOH]^+$ (7), 322 $[M - CH_2CO - AcOH]^+$ (5), 304 $[M - 2AcOH]^+$ (8), 278 $[M - 2AcOH - CO_2]^+$ (30), 245 $[M - 2AcOH - AcO]^+$ (13). ESI-MS: *m*/*z* 463 $[M + K]^+$, 447 $[M + Na]^+$, 425 $[M + H]^+$.

Seed Germination Assay. The toxicity of extracts, fractions, and pure compounds was assessed as the ability to inhibit the germination of stimulated *O. ramosa* seeds, following a procedure already described (*6*). *O. ramosa* seeds were surface sterilized, rinsed with sterile tap water, and placed on wet glass microfiber filters (GF/A Whatman) in Petri dishes. The seeds were kept at 26 °C in the dark for 3 weeks. The filters were then cut into small pieces, each containing around 100 seeds. The pieces were placed on another filter moistened with 2 mL of the assay solution and 5 μg/mL synthetic stimulant (GR24) and kept at 25 °C in the dark. Pure compounds, fractions, and extracts from liquid cultures were dissolved in methanol. Each assay solution contained a final concentration of 0.5% methanol. Crude extracts and fractions were assayed at a final concentration of 100 μg/mL, whereas the pure metabolites were assayed at concentrations between 100 and 0.1 μM. After 4 days, the number of germinated seeds was determined, compared with that of the control, prepared with the same procedure as the treatments but without the culture extracts, and expressed as percentage of inhibition of seed germination.

Table 1. Concentration of Metabolites Produced by *M. verrucaria* or *F. compactum* in Culture Filtrate, and Their Phytotoxicity and Zootoxicity, Assayed on *O. ramosa* Seeds and *A. salina* Brine Shrimps

		activity							
compound[d]	production,[a] mM	*P*,[b] 100 μM	*Z*,[c] 100 μM	*P*, 10 μM	*Z*, 10 μM	*P*, 1 μM	*Z*, 1 μM	*P*, 0.1 μM	*Z*, 0.1 μM
isotrichoverrin B	3.01	100	100	66	67	0	15	0	8
neosolaniol monoacetate	1.65	100	100	100	100	100	41	0	8
roridin A	16.20	100	100	100	100	100	76	nt[e]	21
trichoverrol B	3.10	100	82	19	15	15	5	0	5
verrucarin A	1.59	100	100	100	100	73	84	nt	68
verrucarin B	0.80	100	nt	100	nt	59	nt	nt	nt
verrucarin E	474.80	3	9	nt	2	nt	3	nt	7
verrucarin L acetate	1.66	100	100	100	100	88	100	24	81
verrucarin M	1.00	100	100	100	100	10	100	3	50

[a] Concentration in the culture filtrate. [b] *P* = phytotoxicity, expressed as inhibition (%) of *O. ramosa* seed germination. [c] *Z* = zootoxicity, expressed as mortality (%) of *A. salina* larvae. [d] Neosolaniol monoacetate is produced only by *F. compactum*; all the other metabolites are produced only by *M. verrucaria*. [e] nt = not tested.

Zootoxicity Assay. The zootoxic activity was evaluated using a biological assay on *Artemia salina* (brine shrimps), as previously described (*8*).

RESULTS

Chemical Identification. The culture filtrates of *M. verrucaria* and *F. compactum* were exhaustively extracted with ethyl acetate, both yielding a brown oil, causing the total inhibition of germination of *O. ramosa* seeds. The crude extracts of *M. verrucaria* (510 mg from 1.5 L of culture filtrates) and *F. compactum* (1.137 g from 11 L of culture filtrates) were fractionated by a combination of CC and preparative TLC as described in detail in the Materials and Methods, yielding several toxic metabolites. Another metabolite was further purified by *M. verrucaria* culture extract, showing no phytotoxic and zootoxic activities. The chemical identification of all the metabolites isolated was essentially performed using physical spectroscopic data in comparison to the data previously reported in the literature. Furthermore, considering that NMR spectrometers with high sensitivity and resolution were used, the chemical shifts of all protons and of the corresponding carbons were assigned to each metabolite. Therefore, the only not described physical and spectroscopic data are reported.

Biological assays. *Seed germination assay.* Except for verrucarin E, which does not belong to the trichothecene group and which was inactive (**Table 1**), all the metabolites assayed at 100 μM caused the total inhibition of the stimulated germination. At 10 μM all the trichothecenes were still highly active, causing total inhibition of seed germination, except for isotrichoverrin B, which was slightly less toxic, and trichoverrol B, which was almost inactive. The toxicity of neosolaniol monoacetate and roridin A was particularly noteworthy at 1 μM, as they caused the total inhibition of seed germination. Verrucarin L acetate, verrucarin A, and verrucarin B were still quite active, all causing more than 50% inhibition of germination.

Zootoxic Assay. In the assay on brine shrimps, at 100 μM all the metabolites caused 100% mortality of larvae, with the exception of inactive verrucarin E (**Table 1**). At 10 μM, trichoverrol B was almost inactive, and isotrichoverrin B proved to be slightly less toxic compared to the other metabolites (except verrucarin E). All were still able to cause total mortality of the shrimp larvae. Some of the toxins assayed were still active at 100 nM (**Table 1**).

DISCUSSION

Verrucarins A, B, M, and L, isotrichoverin, and trichoverrol B (**1**−**4**, **6**, and **7**, respectively, **Figure 1**) were isolated from the organic crude extract of culture filtrates of *M. verrucaria* in very low amounts (between 0.8 and 3.1 μM) (**Table 1**) together with roridin A (**5**) and verrucarin E (**8**) (around 16 and 474 μM, respectively) (**Figures 1** and **2**), the latter being the main metabolite. To have sufficient amounts of the extracts for the chemical and biological characterization of the above-mentioned metabolites, a total of more than 10 L of culture filtrates was produced.

Although these metabolites have already been reported, and because reference samples were not available, the chemical identification of verrucarins A, B, L acetate, M, and E, roridin A, isotrichoverin B, and trichoverrol B was performed using essentially spectroscopic and spectrometric methods and comparing the data obtained with those already reported in the literature (*12*−*17*).

The structure of verrucarin L acetate was definitely attributed by the results of a COSY spectrum. In particular, the proton (H-8) linked to the acetoxylated secondary carbon, which appeared as a broad singlet at δ 5.2, coupled with the two protons of the adjacent methylene (H_2C-7) group, resonating as a multiplet at δ 2.20. On the basis of this result, the possible alternative structure showing the acetoxy group at C-3 of the trichothecene ring system was ruled out.

The structure attributed to verrucarin E, which is a 2,4-disubstituted pyrrole, was confirmed by X-ray analysis, recording crystal data identical to those previously described (*10*).

The main toxic metabolite produced by *F. compactum* was isolated, from the organic extracts of the fungal culture filtrates, by a combination of CC and preparative TLC. It was identified as neosolaniol monacetate (**9**) (8.0 mg, 0.7 mg/L) (**Figure 2**) using spectroscopic methods and comparing the data with those already reported in the literature (*17*).

Verrucarins A, B M, and L acetate belong to a subgroup of macrocyclic trichothecenes having a differently functionalized lactone ring located between C-4 and C-15. This macrocycle was substantially different and open, respectively, in roridin A, and isotrichoverrin B and trichoverrol B, which belong to two other subgroups of the macrocyclic trichothecene family (*12*−*14*). However, this is the first time the above-mentioned metabolites are reported to be produced by a strain of *M. verrucaria* isolated from infected tissues of *O. ramosa*. Their potential practical application as natural herbicides for the management of seed germination is possible. Some of these toxins (verrucarins A and B and isotrichoverrin B) were more recently described as metabolites produced in liquid and solid cultures from a strain of *M. verrucaria*, a fungus proposed for

biological control of kudzu, an exotic invasive weed responsible for massive economic and aesthetic losses in the southern U.S. (*18*).

Also in the case of *F. compactum*, neosolaniol monoacetate was already known as a trichotechene produced by different species of *Fusarium* (*17*) including *F. compactum* (*19*), but this represents the first report of its isolation from the liquid culture of a strain of *F. compactum* isolated from infected tissues of *O. ramosa*, and evaluation of its activity on inhibition of *O. ramosa* seed germination.

The trichothecenes are a family of tetracyclic sesquiterpenoid substances produced by several fungal species. More than 100 compounds are known, and cause a wide variety of biological effects owing to the diversity of chemical structures within the group. Trichothecenes are also quite phytotoxic, and the macrocyclics particularly so. For example, in the wheat coleoptile growth inhibitory assay, verrucarin A exhibits activity at a very low level (10^{-8} M) (*20*). Macrocyclic trichothecenes have also been reported to cause increased cellular leakage, growth inhibition, and chlorophyll loss when tested in duckweed (*Lemna pausicostata*) and kudzu (*Pueraria lobata*) (*21*). They all are potent inhibitors of protein synthesis in eucaryotic cells, acting by interfering with peptidyl transferase activity (*22*). Their possible involvement in plant disease development as virulence factors has also been studied (*23*). Although inhibition of seed germination of many plant species (i.e., broccoli, carrot, radish, and turnip) by macrocyclic trichothecenes has already been reported (*24*, *25*), this is the first report of the inhibitory effect of these metabolites to parasitic plant seeds. Furthermore, this toxic effect to seeds is observed at concentrations lower than those reported for cultivated plant seeds (2×10^{-6} M), and at this concentration some seeds (e.g., lettuce, barley, tomato, wheat) proved to be almost resistant (*24*). The results are in general agreement with previous studies carried out using fungal toxins on germination of seeds of *Striga hermonthica* (*26*) and of *O. ramosa* (*6*). In both those studies, among the several toxins tested, all the trichothecenes proved to be highly toxic when assayed for the ability to inhibit the germination of stimulated seeds of parasitic plants. The present results confirm the stronger phytotoxicity of the macrocyclic trichothecenes (acting at 0.1 μM) compared to other trichothecenes. All the compounds assayed showed, as well as all tichothecenes, the presence of an epoxy group, which plays an important role in the biological activity of some classes of naturally occurring compounds (*27*, *28*). As already known, these toxins are powerful mammalian mycotoxins, and the risks of introducing mammalian toxic compounds into the environment should be carefully determined. Even if the evaluation of the in vitro activity of dangerous metabolites represents a preliminary assessment of the risk due to the introduction of mycoherbicides into the environment, the "real" fate of those metabolites when applied in the environment should be carefully ascertained. This would prevent contaminations that would have a great impact on environment/public perceptions, but also prevent useful compounds and producer microorganisms from being discarded because of concerns for their use. For example, neither intact macrocyclic trichothecenes nor toxic metabolites in plant tissues after treatment of kudzu (*Pueraria montana*) with the proposed mycoherbicide *M. verrucaria* could be detected (*18*). It is interesting to observe that the phytotoxicity to *O. ramosa* seeds and zootoxicity to larvae seem unrelated. In fact, at 1 μM there were differences in toxicity, with neosolaniol monoacetate and roridin A being more phytotoxic, and verrucarin L acetate and verrucarin M being more zootoxic.

Considering the efficacy of some toxins at very low concentrations, the possibility of using fungal toxins as natural herbicides to inhibit germination of parasitic plant seeds seems to be not so remote. Many toxins are not selective, being able to cause the same toxic effects both on host and on nonhost plants. For this reason, the toxicity to crop plants has to be determined, even if the application at very low concentration and their quick degradation after inhibition of seed germination could avoid toxic effects. Fungal culture extracts could be an interesting source of new compounds acting as natural and original herbicides. Crude extracts could also be used, after the toxin content has been ascertained. For example, in the case of *M. verrucaria* extract, considering that it contains several active compounds in similar concentrations, its direct use could strongly reduce the cost of its production, avoiding the expensive purification steps, but without reducing its efficacy.

From a practical point of view, considering that most of the crop plants parasitized by broomrape are irrigated, toxins could be introduced by drip irrigation systems, in very low amounts near the host roots. This could prevent the germination of the seeds only where needed, preventing attachment of haustorium to the host roots. This should minimize environmental risks, reducing the amount applied and avoiding toxin dispersal. Furthermore, being natural, those metabolites could be easily bioinactivated soon after their application, reducing risks of drifting and dispersal. Further studies are in progress to assess the stability and the movement of the most active compounds into the soil.

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