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An original approach was developed for the chemical and biological investigation of zone lines formed by the confrontation of fungi growing in confined spaces. Two wood-decaying fungi involved in esca disease, *Eutypa lata* and *Botryosphaeria obtusa*, were grown in Petri dishes. Metabolic profiles of pure fungal strains and confrontation zones were differentially analyzed by ultrahigh pressure liquid chromatography coupled to time-of-flight mass spectrometry (UHPLC/TOFMS). Selected metabolites induced by the confrontation were isolated and characterized by capillary NMR (CapNMR) at the submilligram level. Fungitoxic and phytotoxic assays were applied to the crude extracts and isolated molecules. While the extracts of pure strains were inactive, the extract from confrontation zones exhibited significant activities. A very strongly induced compound, *O*-methylmellein, may explain these toxic properties. The developed approach demonstrates the use of fungal confrontations as an original source of bioactive molecules and gives new insights into the study of esca disease.

KEYWORDS: UHPLC/TOFMS; CapNMR; esca; fungi; *Eutypa lata*; *Botryosphaeria obtusa*; mycelial interaction; zone lines; mycoalexins

INTRODUCTION

Esca apoplectic disease of grapevine is due to a complex of fungi, whose species composition and colonization processes are currently not fully understood. The disease is present worldwide and causes important economic losses to growers who must replace infested vines. Currently, although the defense mechanisms against pathogenic microorganisms have been extensively studied (1, 2), no efficient treatment exists against esca. The cause of the disease has been thought to be the conjunction of physiological misbalance of the plant with the presence of fungi. As reported by Graniti et al. (3), esca is not due to a single pathogen but the combination of different fungi. The mycological complexity of esca has led to numerous studies of the epidemiological aspects of the disease. Previous studies on different grapevine cultivars and from different areas reported the presence of 11 most frequent fungal species in France (4), 5 in Italy (5, 6), and 4 in Germany (7). The six most frequent fungi isolated in Switzerland (8) are the same as those in other countries, namely, *Phaeomoniella chlamydospora*, *Phaeoacremonium angustius*, *Eutypa lata*, *Botryosphaeria obtusa*, *Phomopsis vitiola*, and *Fomitiporia mediterranea*. Most of these species are able to colonize and degrade wood constituents, including lignin, leading to the active degradation of sieve elements. Besides this, fungi in close proximity to each other can interact in different ways, namely, in mutualistic, neutralistic, or competitive interactions (9). Competitive mycelial interactions are very important in the overall development of fungal communities in wood. It has been shown that secondary metabolites of some wood-decaying fungi can act as total inhibitors or stimulators of the growth of other wood-decaying fungi (10). In grapevine, fungi that are in competition can form zone lines, also defined as confrontation or barrage zones (Figure 1), which are morphologically different from pure...
The fungi involved mutually inhibit each other and cannot overgrow their opponent (11). Peiris et al. (12) have recently demonstrated the interest of studying metabolite profiles of mycelial fronts between various oak decomposer basidiomycetes using a GC-MS metabolomic-based approach. However, to date, very little attention has been paid to competition phenomena between fungi for a substrate, as a source of phytotoxic or antifungal substances, or disease markers (13). Their chemical analysis in wood samples presents many constraints, such as the difficulty of ensuring the purity of the confronted strains or the precise excision of the zone lines produced. Furthermore, interference from wood constituents can complicate the preliminary analyses, and the amount of trunk necessary to produce enough fungi is prohibitive.

For these reasons, in this study, fungi were grown in solid media using Petri dishes to investigate the production of mycoalexins (i.e., defense compounds synthesized by fungi under attack, analogous with phytoalexins) (14) resulting from competitive interactions. Two of the fungi mentioned above, namely, *Eutypa lata* and *Botryosphaeria obtusa*, were confronted, and a global strategy of investigation including characterization of the mycoalexins and preliminary assessment of their biological activity was developed. Numerous secondary metabolites produced by *Eutypa lata* alone and implicated in grapevine disease have been reported (15–20). Some phytotoxic compounds have also been described in *Botryosphaeria obtusa* (21).

Because of the restricted amounts of material that are available from Petri dishes, very sensitive analytical techniques had to be selected for metabolite profiling and complete de novo characterization of mycoalexins. In this respect, ultrahigh pressure liquid chromatography (22) coupled to time-of-flight mass spectrometry (UHPLC/TOFMS) is known to provide high resolution profiling of complex biological matrices (23, 24). Additionally, the at-line structural determination of natural products at the microgram level (25–27) can be performed by micro NMR methods designed for mass limited samples such as capillary NMR (CapNMR) (28). A complete integration of these methods in complement to rapid bioassays of original mycoalexins is presented.

**MATERIALS AND METHODS**

**Chemicals.** Solvents used for extraction were methanol ACS-ISO for analysis (Carlo Erba SA, Val de Reuil, France), chloroform GR for analysis (Merck, Darmstadt, Germany), and nanopure water (Millipore). ULC/MS grade acetonitrile (CH3CN) and water from Biosolve (Valkenswaard, The Netherlands) were used for the UHPLC/TOFMS analyses. For the HPLC isolation step, solvents were HPLC grade CH3CN Chromanorm from VWR (Leuven, Belgium) and nanopure water. For the NMR experiments, methanol-d4 (CD3OD; 99.8% atom deuterium) was obtained from Armar Chemicals (Buchs, Switzerland).

**Fungal Growth.** Fungi were grown on potato dextrose agar (PDA, Difco) under alternating light and dark periods (12 h/12 h) at 21 °C. The strains of *Botryosphaeria obtusa* and *Eutypa lata* used in this study were isolated from the experimental vineyards of ACW Changins (Switzerland). Growth speed of each fungus was measured each day on PDA for two weeks under the same conditions. Fully developed colonies were used to inoculate 44 Petri dishes (145 mm diam.) using 3 mm diameter fungal chips of the different species on each side of the plate according to the growth speed of each fungus. Cultures were grown under identical conditions until formation of the fungal confrontation zone. Additionally, each fungus was grown separately on PDA in four replicates under the same conditions.

**Extraction Procedure.** Confrontation zones of 1.5 mm wide were cut and excised using a razor blade, then placed in a vessel with distilled water (100 mL water/100 g extracted agar). Agar media from uninoculated PDA plates and from plates inoculated with each one of both fungal species were used as controls. This initial material was crushed in a Primax blender (Müller & Krempel AG, Bülach, Switzerland) for 5 min and centrifuged (3500g, 15 min, 4 °C). The pellet was washed with water and centrifuged (3500g, 30 min, 4 °C). The extraction was performed in chloroform/methanol/water (64:36:8, v/v) in the dark under agitation (2 h, 4 °C), followed by filtration through LS 14 1/2filter paper of 500 mm diameter (Schleicher & Schuell GmbH, Dassel, Germany). The filtrate was then dried under vacuum with a rotary evaporator, and the resulting residue was stored at −27 °C until use. Before analysis, a purification procedure using a Sep Pak Vac SPE C18 cartridge (12 cc, 2 g) was performed. MeOH/H2O (90: 10, v/v) was used for loading and elution to retain very apolar compounds that would be incompatible with reverse-phase chromatography.

**UHPLC/TOFMS Experiments.** UHPLC/TOFMS analyses were performed on a Micromass-LCT Premier Time of Flight mass spectrometer from Waters (Milford, MA, USA) with an electrospray (ESI) interface coupled with an Acquity UPLC Premier system from Waters. Detection was performed in positive (PI) and negative ion (NI) modes during the same analysis in the range m/z 100–1000 in centered mode with a scan time of 0.2 s and an interscan delay of 0.3 s for polarity switching. ESI conditions in PI and NI modes were capillary voltage 2800 V, cone voltage 40 V, source temperature 120 °C, desolvation temperature 250 °C, cone gas flow 20 L/h, and desolvation gas flow 800 L/h. For internal calibration, a solution of leucine–enkephalin from Sigma-Aldrich (Steinheim, Germany) at 5 µg/mL was infused through the lockmass probe at a flow rate of 5 µL/min, using a second Shimadzu LC-10ADvp LC pump (Duisburg, Germany). The separation was performed on a 150 mm × 2.1 mm i.d., 1.7 µm, Acquity BEH C18 UPLC column (Waters) in the gradient mode at a flow rate of 0.3 mL/min with the following solvent system: A = 0.1 vol % formic acid–water, B = 0.1 vol % formic acid–acetonitrile; 2–98% B in 30.0 min. The temperature was set at 30 °C. The injected volume was 5 µL.

**LC-MS-Monitored Semipreparative Isolation.** The analyses were performed on a Finnigan MAT (San Jose, CA, USA) triple quadrupole mass spectrometer (TSQ700) equipped with a Finnigan ESI interface and coupled to a Varian modular HPLC system (Palo Alto, CA, USA).
Investigation of Induced Metabolites in Fungal Confrontation Zones

RESULTS AND DISCUSSION

The global strategy used in this study for the chemical and biological investigation of confrontation zones between fungi is presented in Figure 2.

Fungal Growth and Sample Preparation. Among all the fungal species identified in esca apolecptic grape woods, those isolated most frequently were selected for confrontation tests. The final choice was based on the ability of the selected fungi (Eutypa lata and Botryosphaeria obtusa) to have a rapid growth rate and to form well-defined confrontation zones. Growth speeds of Botryosphaeria obtusa (0.8 mm/h) and Eutypa lata (0.2 mm/h) were measured to determine which of the two fungi had the slowest growth and needed to be inoculated first. This point was crucial to obtain broad and well-defined confrontation zones (Figure 1). In the case of this specific confrontation, Botryosphaeria obtusa was inoculated 5 days after Eutypa lata. The whole culture medium including fungi and agar, representing 44 Petri dishes for a total mass of 750 g of material, was extracted in quadruplicate (4 × 11 dishes). The yield was thereby calculated from the ratio between the initial mass of agar medium and the mass of final extract residue. Sample preparation was kept to a minimum in view of the analysis of the widest possible range of compounds; a simple SPE procedure using a C18-based cartridge was undertaken to retain apolar compounds that could contaminate the UPLC column and instrumentation. This step was determined to be mandatory to avoid carryover and cross-contamination effects. The four replicates represented a total of 170 mg of enriched extract corresponding to a yield of 0.022 ± 0.01%.

UHPLC/TOF-MS Profiling of Fungal Confrontation Zones. Since fungal strains represent biological samples of complex metabolite composition, UHPLC was selected for the differential analysis of the confrontation zone and pure strain extracts. Separation conditions were chosen to maximize peak capacity...
The amounts injected and the intensity thresholds are identical for all extracts. Pure strain of *Botryosphaeria obtusa* (UHPLC/TOFMS base peak intensity (BPI) chromatograms of the extracts recorded in positive ion mode. (Figure 3)

In the BPI chromatogram recorded in PI mode, about 150 peaks were detected in each pure strain, and over 200 peaks in the confrontation zone. This indicated that numerous unidentified compounds were strongly induced in the region where the fungi came into competition. The differences between the various situations were highly significant. Approximately 60% of the induced metabolites were not detected in the pure strains; they were most likely synthesized de novo as a result of the confrontation. The others were already present in the pure strains but at a much lower level than in the confrontation zone. In particular, some polar substances of low molecular weight recorded in the PI mode (compounds 1–4) (Figure 3C) exhibited a very strong MS response in the confrontation extract. Since all of the substances toxic for grapevine reported to date in *Eutypa lata* or *Botryosphaeria obtusa* are small, polar molecules (MW 122–266 Da, e.g., vinyl-acetylene derivatives mentioned above), compounds 1–4 presented a particular interest and were thus selected for further experiments. Compound 1 had a [M + H]+ ion at m/z 193.0870 corresponding to the molecular formula C_{11}H_{13}O_{4} (error 2.6 ppm). The double bond equivalency (DBE) of 6 meant that the molecule contained 6 rings and/or double bonds. Compounds 2–4 all presented a [M + H]+ ion at m/z 209.0822 (C_{11}H_{15}O_{5}, error 3.8 ppm). They only differed by one oxygen atom from 1 and could be potential hydroxylated derivatives of 1. However, it was not possible to determine the complete structure of these molecules on the basis of only TOFMS information. Therefore, their microisolation by semipreparative LC was undertaken to enable complete characterization by NMR.

**MS-Monitored Semipreparative LC Isolation of Induced Metabolites.** Because of the small amount of extract available (four extracts representing a total of 170 mg after pooling), the sample consumption during the optimization of the isolation method had to be as low as possible. Thus, a geometrical transfer from the analytical to semipreparative scales was performed to maintain identical selectivity and chromatographic profile (32). The stationary phase chemistry and mobile phase composition were kept identical, while the gradient time, flow rate, and injected volume were adapted to the column geometry. A splitter (for MS detection) and a fraction collector were used. About 160 mg of extract was separated in four successive injections. Two analyses were recorded in each ionization mode. The excellent repeatability of retention times enabled a precise

(24): an average retention factor in gradient analysis (kₐ) of 3 and a peak capacity around 300 were obtained. TOFMS was chosen as a detector for its very high sensitivity in scan mode and for its high mass accuracy, which could give some structural information by providing molecular formulas of the compounds of interest.

Samples were analyzed simultaneously in both PI and NI modes in quadruplicate to detect a maximal number of metabolites and evaluate the consistency of their occurrence. **Figure 3** presents the base peak intensity (BPI) chromatograms of *Eutypa lata*, *Botryosphaeria obtusa*, and confrontation zone extracts, recorded in PI mode. In all cases, the quantity injected was equivalent. In a first step, the data obtained for the *Eutypa lata* and *Botryosphaeria obtusa* chromatograms were processed for the dereplication of known secondary metabolites. The determination of their presence was based on literature data and exact mass measurements by TOFMS (<5 ppm) providing molecular formulas. Although an unambiguous confirmation of their occurrence was not possible in the absence of available standards, six reported vinyl-acetylene derivatives were tentatively identified in the *Eutypa lata* extract, namely, eutypine (MW 186.0681), eutypinol, and its isomer eulatinol (MW 188.0837), siccacne (MW 174.0681), methylteutypinol (MW 202.0994), eutypinic acid (MW 202.0630), and a vinyl-allene derivative (MW 194.0943) (20). It has to be noted that over 20 compounds have been described in *Eutypa lata*; however, metabolite patterns vary according to the origin of the strain (16). This might explain why only part of these compounds were detected. In the *Botryosphaeria obtusa* extract, only two putative isomers of hydroxymellein (MW 194.0579) could be identified.

All of the described compounds appeared in the chromatographic zone of high polarity (0–15 min) and were also present in the confrontation zone extract; however, for these metabolites, no significant induction was observed. As presented in **Figure 3** (PI mode), the confrontation zone extract was obviously richer in compounds than the pure strain extracts, particularly in the high polarity domain of the chromatogram (0–10 min). This observation was confirmed in the NI mode (data not shown). In the BPI chromatogram recorded in PI mode, about 150 peaks were detected in each pure strain, and over 200 peaks in the confrontation zone. This indicated that numerous unidentified compounds were strongly induced in the region where the fungi came into competition. The differences between the various situations were highly significant. Approximately 60% of the induced metabolites were not detected in the pure strains; they were most likely synthesized de novo as a result of the confrontation. The others were already present in the pure strains but at a much lower level than in the confrontation zone. In particular, some polar substances of low molecular weight recorded in the PI mode (compounds 1–4) (Figure 3C) exhibited a very strong MS response in the confrontation extract. Since all of the substances toxic for grapevine reported to date in *Eutypa lata* or *Botryosphaeria obtusa* are small, polar molecules (MW 122–266 Da, e.g., vinyl-acetylene derivatives mentioned above), compounds 1–4 presented a particular interest and were thus selected for further experiments. Compound 1 had a [M + H]+ ion at m/z 193.0870 corresponding to the molecular formula C_{11}H_{13}O_{4} (error 2.6 ppm). The double bond equivalency (DBE) of 6 meant that the molecule contained 6 rings and/or double bonds. Compounds 2–4 all presented a [M + H]+ ion at m/z 209.0822 (C_{11}H_{15}O_{5}, error 3.8 ppm). They only differed by one oxygen atom from 1 and could be potential hydroxylated derivatives of 1. However, it was not possible to determine the complete structure of these molecules on the basis of only TOFMS information. Therefore, their microisolation by semipreparative LC was undertaken to enable complete characterization by NMR.

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**Figure 3.** UHPLC/TOFMS base peak intensity (BPI) chromatograms of the extracts recorded in positive ion mode. (A) Pure strain of *Eutypa lata*. (B) Pure strain of *Botryosphaeria obtusa*. (C) Mycelial confrontation zone. Compounds 1–4 are highlighted in the different extracts when they are detectable. The amounts injected and the intensity thresholds are identical for all extracts.
pooling of the fractions obtained from the different injections. Because of the MS monitoring and the selectivity identical to that achieved in UHPLC/TOFMS, the compounds of interest were easily localized. Compound 1 was collected in fractions 23–25 (RT 22–25 min). Compounds 2 and 3 were obtained together from fraction 13 (RT 12–13 min). Compound 4 was present in fraction 16 (RT 15–16 min). Their purity was controlled by UHPLC/TOFMS. Compound 1 was most likely the major product of the extract since the corresponding peak was the only one that saturated the semipreparative column (Figure 4A); about 1.5 mg was isolated. As it was approximately 100 times less abundant in the pure strains, this compound was likely to play a major role in the competition between both species studied. For 2, 3 and 4, about 300–400 µg was obtained. Such amounts were not sufficient to perform 1D and 2D NMR experiments with conventional instruments. Thus, capillary NMR (CapNMR) was selected for the structural elucidation of 1–4. Because of its low active volume, the CapNMR probe is particularly appropriate for the analysis of limited sample amounts (28). The fractions containing 1–4 were dried and dissolved in 7 µL of CD$_3$OD before CapNMR experiments.

**Figure 4.** (A) LC-MS semipreparative chromatogram of the confrontation zone extract recorded in positive ion mode. (B) $^1$H CapNMR spectrum of the mixture of 2 and 3. Inset in B: Magnification of the peaks corresponding to the methyl groups, illustrating the difference in signal intensities between both isomers. (C) $^1$H CapNMR spectrum of 1. (D) gHMBC CapNMR spectrum of 1. Circles: residual HSQC correlations. * Impurities.

Structural Elucidation of the Isolated Mycoalexins by CapNMR. Characterization of Compound 1. Before performing CapNMR, a hydrogen/deuterium (H/D) exchange experiment was conducted on purified compound 1 (C$_{11}$H$_{12}$O$_3$) using TOFMS and indicated that no labile hydrogen atom was bound to one of the three oxygen atoms present. 1D and 2D CapNMR spectra revealed that 1 held a trisubstituted aromatic ring (3 adjacent protons between δ 6.91–7.55 ppm) with one of the substituents being an O-methoxy group (singlet at δ 3.91 ppm) and another an ester group (long-range HMBC correlation between H-7 and C-1 at δ 164.1 ppm; Figure 4D). Additionally, a CH$_3$-CH-CH$_3$ chain being part of a methylated pyran-2-one cycle attached to the aromatic ring was present. The protons of the methylene group were diastereotopic (δ 2.89 and 2.98 ppm). These NMR data were in agreement with those reported by
Dimitriadis et al. for O-methylmellein (33), and 1 was identified accordingly (Figure 5). The complete 1H and 13C signal assignments for 1 are reported in Table 1, for comparison with 2–4 and because data were acquired in a different solvent (CD3OD). Figure 4C presents the 1H CapNMR spectrum and Figure 4D the gHMB spectrum of O-methylmellein. It clearly indicates the very high degree of purity obtained by the single preseparation isolation step. The very small amount obtained in this study for 1 prevented the determination of its absolute configuration. Melleins and substituted melleins are known metabolites of many fungi, including Botryosphaeria obtusa (21) and Eutypa lata (34) with an R configuration at C-3. Thus, it was assumed that 1 was R-(−)-O-methylmellein and that the other derivatives identified shared the same core structure (Figure 5). No biological activity has previously been described for O-methylmellein, but its very strong induction in the confrontation zone suggests a putative role in the competition between the confronted fungi.

Characterization of Compounds 2, 3, and 4. Because of the mass difference corresponding to an oxygen atom, 2–4 were presumed to be hydroxylated isomers of 1. H/D exchange experiments on 2–4 revealed one labile hydrogen atom and confirmed a possible hydroxyl group of 1.

With the generic gradient and fractionation time used for semipreparative isolation, 2 and 3 were obtained in the same fraction. Figure 4B presents the 1H CapNMR spectra of the mixture of 2 and 3. Both isomers exhibited very similar 1H spectra, closely related to that of 1. Nevertheless, the deconvolution of their respective signals was possible since slight differences in chemical shifts and an integration ratio of about 1/2 existed between them (Figure 4B, inset). The complete 1H and 13C signal assignments for 2 and 3 are reported in Table 1. The only significant difference from 1 was the downfield shift of C-4 (δ 67.3 and 69.0 ppm for 2 and 3, respectively, vs δ 35.4 ppm for 1) and its attached proton (H-4 at δ 4.56 and 4.51 ppm for 2 and 3, respectively, vs H-4αβ at δ 2.89 and 2.98 ppm for 1). This indicated the presence of a hydroxyl group at C-4 for both 2 and 3, which was confirmed by a gCOSY correlation between H-3 and H-4. The distinction between 2 and 3 could be made from the different coupling constants between H-3 and H-4. For 3, a H-3/H-4 coupling constant of 8.3 Hz was observed, indicative of an axial–axial coupling. For 2, no coupling constant could be detected, suggesting either axial–equatorial or equatorial–equatorial couplings. As for 1, the absolute configuration could not be determined by NMR.

Compound 4 was found to be a positional isomer of 2 and 3 since the hydroxyl group was bound to C-5 instead of C-4. This was shown by a strong downfield shift of C-5 at δ 147.1 ppm compared to 1, 2, and 3 and by the absence of the corresponding aromatic H-5 proton. The position of H-7 remained unchanged in 1–4 on the basis of a NOESY correlation with the protons of the methoxy group. Since, in the case of 4, H-7 exhibited an ortho coupling with H-6, the hydroxylation position was ascertained as C-5 (Table 1).

In Vitro Antifungal and Phytophotoxicity Assays. The biological activity of crude extracts and pure compounds (O-methylmellein and its hydroxylated forms) was evaluated in representative fungi and plants, on the basis of developmental criteria of germination and growth. First, the ability to specifically inhibit the development of Botryosphaeria obtusa and Eutypa lata was considered by microscopic observation of solid media cultures. Remarkably, O-methylmellein (25 µg spotted) showed a very high fungitoxic activity on the development of Botryosphaeria obtusa. The mycelial growth was completely stopped, to the same extent as the fungicide control. However, interestingly, no effect was detected on Eutypa lata. Hydroxylated forms of O-methylmellein at identical concentrations did not exhibit any effect on the growth of Eutypa lata or Botryosphaeria obtusa. These compounds are present in high concentration in confrontation zones and absent from pure strains. Their concomitant appearance in the mycelial front with the active O-methylmellein might indicate the metabolism of the latter by Botryosphaeria obtusa, as part of a putative detoxification process. Indeed it has been reported that, apart from metabolite induction, numerous metabolic changes occur during mycelial interactions (35). Therefore, different enzymes implicated in detoxification mechanisms might be activated, especially in the case of fungi involved in the degradation of wood constituents (36). Total extracts from pure fungal cultures and the confrontation zone did not show any effect on the growth speed of Botryosphaeria obtusa and Eutypa lata (25 and 200 µg spotted). Due to the low amount of extract available, higher concentrations could not be tested.

In order to estimate the overall fungitoxic and phytotoxic activity of the extracts and compounds described above, the inhibitory activity on the germination of Botrytis cinerea conidia and on the germination and growth rate of garden cress (Lepidium sativum) was evaluated. In the case of Botrytis cinerea, the inhibition was assessed microscopically as was done for the tests on Eutypa lata and Botryosphaeria obtusa. Here also, the extracts from pure cultures (25 and 200 µg spotted) did not show any activity. However, the confrontation zone extract significantly affected germination. The conidia germinated by producing very short (1/10 of the control) and swollen germ tubes. In the case of the isolated compounds (25 µg spotted), O-methylmellein presented a high inhibitory activity on germination, showing only the formation of the initial germ tubes, while its hydroxylated metabolites were inefficient.

The phytotoxicity was evaluated using an original assay performed on garden cress on agar medium (Figure 6), partly derived from the work of Delabays et al. (31). Only O-methylmellein showed a strong antigerminative effect at very low concentration (0.001%), more pronounced than the positive control, artemisinin (0.033%). The hydroxylated forms did not show any effect, with the growth rate of the aerial part and roots identical to the negative water control. The phytotoxicity of the crude extracts was tested at different concentrations. Only the extract resulting from the confrontation area showed a significant effect on growth, resulting in the development of the aerial part six times lower than the water control at a concentration of 0.05% (Figure 6). The combined action of both fungi appears thus to increase the toxicity toward garden cress. The strong antigerminative effects observed suggest that the fight between fungi might affect the primary metabolism of the plant.
Table 1. CapNMR Signal Assignments for O-Methylmellein (1), Diastereoisomers of 4-Hydroxy-8-O-methylmelleine (2 and 3), and 5-Hydroxy-8-O-methylmelleine (4) in CD$_3$OD$^a$

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<td>55.3</td>
<td>5.32s</td>
<td>55.4</td>
<td>3.92s</td>
<td>55.4</td>
<td>3.84s</td>
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<tr>
<td>8a</td>
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<td>111.8</td>
<td></td>
<td>111.3</td>
<td></td>
<td>113.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$s, singlet; $d$, doublet; $t$, triplet; $q$, quadruplet; $m$, multiplet. $^b$Reference for protons (methanol $\delta$ H 3.31 ppm). $^c$Reference for carbons (methanol $\delta$ C 49.0 ppm).

Figure 6. Phytotoxic effects of crude extracts and pure compounds issued from fungal cultures on germination and growth of garden cress: aerial part growth in centimeters is indicated for each case. (Lane A) Positive control: artemisinin (0.033%). (Lane B) O-Methylmellein (1) (0.001%). (Lane C) 4-Hydroxy-8-O-methylmelleine (2 and 3) (0.003%). (Lane D) Negative control (water). (Lane E) Crude extract from pure strains of Botryosphaeria obtusa at 0.1%. (Lane F) Crude extract from pure strains of Eutypa lata at 0.1%. (Lane G–I) Crude extracts from the confrontation zone at 0.05, 0.025, and 0.0125%, respectively.

Consequently, toxicity on grapevine seems highly probable, potentially related to $O$-methylmellein. Further tests on the germination of vine and on wood of mature specimens should be conducted to confirm this hypothesis but will necessitate the production of higher amounts of bioactive extracts and related mycoalexins.

In conclusion, these results clearly indicate that some of the metabolic changes recorded within the confrontation zone play an important role in the specific growth inhibition of Botryosphaeria obtusa and more generally on the toxic activity toward other fungi or plants. The metabolite induction phenomena and related bioactivities evidenced shed light on the complex processes implicated in esca disease. Moreover, the herbicide potential of the mycoalexins studied may open new areas of applications in agronomy.

While the ability of fungi to produce active secondary metabolites has been extensively investigated (13), little attention has been paid to the induction of compounds in confrontation zones. The present work has demonstrated the complexity and richness of such zones and their potential for the discovery of new bioactive natural products. Other fungi should be investigated and biological assays conducted to potentially identify, for instance, new lead compounds also active against human pathogens.

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LITERATURE CITED
