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ARTICLE

Metabolite Composition and Bioactivity of *Rhizoctonia solani* Sclerotial Exudates

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Sclerotia are vegetative structures that play a major role in survival of fungi under adverse conditions. The sclerotia of the plant pathogen *Rhizoctonia solani* AG2-2 IIIB exude liquid brown droplets that were evaluated for their bioactivity and toxicity against microorganisms and plant species. Also, their metabolic composition was analyzed by integrating Fourier transform ion cyclotron resonance−mass spectrometry (FT-ICR/MS), gas chromatography−MS (GC/MS), and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. The results showed that exudates are complex mixtures composed of phenolics (17.40%), carboxylic acids (12.79%), carbohydrates (6.08%), fatty acids (3.78%), and amino acids (3.47%). The presence of such metabolites contributed to their antifungal and phytotoxic activities. The biological interpretation of the results highly suggests that the exudates not only have multiple roles in fungal physiology but also are a potential bioactive source with moderate toxicity. Our findings show with certainty that the integration of different analytical platforms is a powerful approach for extracting the maximum and reliable information on the metabolic composition of complex biological samples.

KEYWORDS: Exudation; Fourier transform ion cyclotron resonance−mass spectrometry (FT-ICR/MS); gas chromatography−mass spectrometry (GC/MS); natural products; nuclear magnetic resonance (NMR) spectroscopy; *Rhizoctonia* solani; sclerotia; toxicity

INTRODUCTION

Many compounds derived from fungi have been exploited in human and veterinary medicine and crop protection (*1*−*4*). Fungal plant pathogens are capable of inducing disease symptoms in their respective hosts by virtue of the phytotoxins that they produce. Some of these compounds or their chemical templates have been successfully developed as crop protection agents (*3*, *4*).

The fruiting structures of various fungi are avoided by fungivorous insects due to the presence of toxic fungal metabolites (*5*). One such fruiting structure is the sclerotium, a reproductive body produced by certain fungi as a mechanism for survival and propagation of the species (*6*). During their dormant phase, sclerotia are exposed to potential predators and parasitic organisms that use the sclerotia as source of food. By analogy to plant seeds, some of which are known to contain toxic metabolites to deter predators, sclerotia represent a substantial metabolic sink of bioactive compounds exhibiting antifungal, antiinsectan and antimicrobial properties that discourage their predation and parasitism (*7*−*10*). Thus, potentially, there is a reservoir of novel biologically important substances awaiting discovery in these structures.

Among the sclerotia-producing fungi is the cosmopolitan soilborne plant pathogen *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris*) that affects a large number of economic crops (*11*−*13*). Commonly occurring metabolites produced in culture filtrates of *R. solani* are phenylacetic acid (PAA) and its hydroxylated derivatives (*14*−*19*), N_b-acetyltryptamine and proline containing dioxopiperazines (*17*), and an unidentified host specific toxin containing a carbohydrate moiety (*18*).

A common phenomenon observed during the early development of sclerotia in many fungal species, including some *R. solani* anastomosis groups, is the exudation of liquid in the form of droplets developing on the surface of sclerotia (*19*−*21*). Analyses of the chemical constituents of sclerotial exudates of *Sclerotium rolfsii* and *Macrophomina phaseolina* revealed the presence of amino acids, sugars, and organic acids (*20*, *22*). The presence of such compounds is thought to be responsible for the sclerotial resistance against microbial attack. For example, the sclerotial exudates of *S. rolfsii* showed antifungal activity against some parasitic and saprophytic fungi (*10*). Nonetheless, clear evidence on the function and the role of exudates in fungal physiology is not yet clear.

Despite these findings, the information on liquid droplets from sclerotia of different fungi is still fragmentary. Furthermore, studies on the chemical composition and bioactivity of exudate droplets of *R. solani* sclerotia, considering the large amounts of exudates formed on the surface of the sclerotia, are nonexistent.

Different analytical platforms have been applied for metabolic analyses of samples of biological origin (*23*−*25*), and each is associated with specific advantages and disadvantages (*26*). Because of the physicochemical properties and complexity of *R. solani* exudates, a comprehensive analysis combined with a

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Table 1. List of Organisms Used To Assess the Bioactivity of Sclerotial Exudates of *Rhizoctonia solani* AG2-2 IIIB Isolate, and the Culture Media/Substrates and Growth Conditions

type of bioassay	organism	culture medium[a] or substrate	temp (°C)
	Plants		
chlorophyll content	Barnyard grass (*Echinochloa crus-galli* L.)	sand	22 ± 1
	Beet (*Beta vulgaris* L.)	sand	
	Duckweed (*Lemna minor* L.)	Steinberg medium	
	Kentucky bluegrass (*Poa pratensis* L.)	sand	
	Velvetleaf (*Abutilon theophrastii*)	sand	
	Wild oat (*Avena sterilis* L.)	sand	
	Wheat (*Triticum aestivum* L.)	sand	
no. of fronds	Duckweed (*Lemna minor* L.)	Steinberg medium	
seed germination	Lettuce (*Lactuca sativa* L.)	water	
	Fungi		
spore germination	*Fusarium sporotrichioides*	PDA	24 ± 1
	Heterosporium solani	V8 agar	
	Phytophthora infestans	V8 agar	
	Stachybotrys elegans	PDA	
	Trichoderma virens	PDA	
	Bacteria		
well diffusion	*Corynebacterium michiganense* pv *michiganense* (Gram +)	LB, LBA	38 ± 1
	Erwinia carotovora subs. *atroseptica* (Gram −)	LB, LBA	28 ± 1
	Escherichia coli DH5α (Gram −)	LB, LBA	38 ± 1
	Streptomyces scabies EF-35 (Gram +)	LB, LBA	28 ± 1
Microtox	*Vibrio fischeri*	reconstitution solution and diluent (Microtox)	

[a] LB, Luria−Bertani; LBA, Luria−Bertani agar; PDA, potato dextrose agar; Steinberg medium (International Organization for Standardization, 2003).

multiprong analytical approach is essential for the detection and identification of a wide range of metabolites. To this end, we set out to apply direct infusion Fourier transform ion cyclotron resonance−mass spectrometry (FT-ICR/MS), which is a superior MS detector to date owing to its great resolution, accuracy, and minimum requirements in sample processing (*27*, *28*). In order to mine the maximum information on the chemical composition of exudates, two complementary platforms to FT-ICR/MS were also applied: gas chromatography−mass spectrometry (GC/MS), which enables chromatographic separation and MS libraries searches based on fragmentation patterns, and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, which provides structural information on compounds.

Here we describe a holistic approach to evaluate the bioactivity of *R. solani* AG2-2 IIIB sclerotial exudates and their role in fungal physiology through comprehensive analysis of their metabolic composition and assessment of their bioactivity against various plant species and microorganisms.

MATERIALS AND METHODS

Chemicals and Reagents. The antibiotic penicillin, deuterium oxide (D_2O, 99.9%) containing trimethylsilyl-2,2,3,3-d_4-propionic acid sodium salt (0.05%), pyridine (99.8%), methoxylamine hydrochloride (98%), and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). Analytical standards were purchased from Restek Corporation (Bellefonte, PA) and methanol and dimethyl sulfoxide (DMSO) from Fisher Scientific Company (Ottawa, ON, Canada). The reconstitution solution and diluent for the Microtox assay were obtained from Azur Environmental (Carlsbad, CA).

Plant Material. The phytotoxicity of exudates was assessed against representative monocotyledonous and dicotyledonous crops and weed species (**Table 1**). Seeds of barnyard grass (*Echinochloa crus-galli* L.), Kentucky bluegrass (*Poa pratensis* L.), velvetleaf (*Abutilon theophrastii*), and wheat (*Triticum aestivum* L.), were provided by courtesy of Prof. A. Watson (McGill University). Seeds of wild oat (*Avena sterilis* L.) were collected from fields in central Greece, whereas those of lettuce (*Lactuca sativa* var. Simpson) were purchased from McKenzie Co. Inc. (Brandon, MB, Canada) and sugar beet (*Beta vulgaris* var. Bronco) seeds were supplied by Hilleshøg (a branch of Syngenta, Landskrona, Sweden). All seeds were stored at 4 °C until further use.

For the development of seedlings, five seeds were planted in plastic pots (6 × 6 × 6 cm) using sterilized sand at 120 °C for 1 h, and incubated in a growth chamber at 22 ± 1 °C, with relative humidity of 85%, and a 14 h photoperiod. The plants were watered every second day. One-week-old seedlings whose root system was excised were used in bioassays.

The ecotoxicological risk assessment of exudates was performed using the aquatic plant *Lemna minor* L., UTCC 490 (University of Toronto Culture Collection, ON, Canada), a model organism for ecotoxicological studies (*29*, *30*). *L. minor* cultures were grown and maintained in plastic beakers on Steinberg medium (*31*) according to the method of Aliferis et al. (*30*).

Microorganisms and Growth Conditions. A highly virulent *Rhizoctonia solani* strain (isolate ATCC 76124), belonging to AG2-2 IIIB, was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained on oat kernels (*32*). The strain is highly pathogenic to its host, sugar beet, causing damping off diseases with 82.5 ± 1.5% of the infected seedlings affected five days after treatment.

Fungal and bacterial species (**Table 1**) that were used for the assessment of the biological activity of exudates were purchased from the Laboratoire de diagnostic en phytoprotection (Sainte-Foy, QC, Canada). *Escherichia coli* DH5α was purchased from Invitrogen (Burlington, ON, Canada). Fungal cultures were maintained on PDA or V8 agar [V8 juice 20% (v/v), Bacto agar 2% (w/v)], and subcultures were made at three-week intervals using a 5 mm plug for the inoculation of the media.

The Microtox assay was carried out using the lyophilized strain NRRL-B-11177 of the bioluminescent bacterium *Vibrio fischeri* (*Photobacterium phosphoreum*) (Azur Environmental).

Collection and Preparation of Sclerotial Exudates for Bioassays. Starter cultures from colonized *R. solani* AG-2 IIIB oat kernels were placed in Petri plates (9 cm in diameter) containing potato dextrose agar (PDA; Becton Dickinson Microbiology Systems, Sparks, MD) and kept in the dark at 24 ± 1 °C. Agar plugs (0.5 cm in diameter) from three-day-old cultures were placed in Petri plates (9 cm in diameter) containing PDA and kept in the dark at 24 ± 1 °C. Subcultures were made at three-week intervals and were regularly observed for sclerotia formation and production of exudates, which are typically produced two weeks after the initial

inoculation of culture medium. The exudates were pipetted from the sclerotial primordia of two-week-old fungal cultures with a 1 mL Gilson pipet and collected into glass beakers of 200 mL capacity. An average of 150 mL of sclerotial exudates were collected from 100 Petri plates and stored at −80 °C until further use. Exudates were dried using a Speedvac AS1010 (Savant Instruments, NY). Working solutions of exudates used in bioassays were prepared by redissolving the appropriate amount of powdered exudates in sterile distilled water or PDB. Solutions were then filter sterilized (MF Millipore GVWP, 0.22 μm) and kept at −80 °C until further use in screw-thread glass vials (2 mL). For the standardization of the bioassays, the concentration of working solutions was expressed as μg of dry weight per mL of the solution (ppm). The pH of exudates was slightly basic, close to 7.5.

Assay for Phytotoxic Activity against *Lemna minor* and Terrestrial Plants. *L. minor* is a valuable organism in assessing the toxicological activity of exudates. Colonies of the same age and overall appearance were selected and placed in plastic 24-well Corning Costar cell culture plates (Sigma-Aldrich Ltd.). One colony was placed in each well containing 1 mL of Steinberg medium or 1 mL of the working solutions of exudates (1,500, 3,000, and 6,000 ppm in Steinberg medium). Measurements were taken 120 h after treatments and the concentrations of exudates that are required to obtain 50% inhibition (IC_{50}) in the number of fronds and chlorophyll content were calculated.

The effect of exudates on young seedlings of various mono- and dicotyledons (**Table 1**) was also studied. One-week-old seedlings, whose root systems were excised, were immersed in 1.5 mL of exudate solution (10,000 ppm, pH 7.5) in Eppendorf tubes. Seedlings immersed in sterile water served as controls and observations were taken 72 h after treatments.

The chlorophyll content was monitored using DMSO as the extracting solvent (*33*) and the total number of fronds (for *L. minor* only) were used as indicator of exudates' phytotoxicity. Absorbance of the extracts was read at 665 nm using a NanoDrop ND-1000 spectrophotometer (Fisher Scientific Company Inc., Ottawa, Canada). The chlorophyll content was expressed as $A_{665}\ g^{-1}$ of fresh weight. Analyses were performed five days after treatments, and each experiment was conducted in triplicate with three replications each.

The effect of exudates on seed germination was conducted as an additional indicator of phytotoxicity. Lettuce (*L. sativa* L.) was used as the bioassay plant because it produces tiny seeds that have the ability to germinate easily and uniformly, and grow relatively quickly. A rapid bioassay determining the concentration required to obtain 50% inhibition of seed germination (IC_{50}) was performed on lettuce seeds using the miniaturized method of Dayan et al. (*34*) which optimizes the use of compounds that are generally available in small quantities. Briefly, three lettuce seeds were added to each well of a 96-well ELISA plate containing 50 μL of working solutions of exudates (2,500, 5,000, and 10,000 ppm) or water serving as control. Seed germination was estimated 72 h after treatments, and the whole experiment was performed three times with three replications each.

Assay of Antifungal Activity. The antifungal activity of exudates was assessed against the conidia of various fungi (**Table 1**). Spore suspensions (50,000 spores mL^{-1}) harvested from three-week-old cultures were prepared in potato dextrose broth (PDB, Difco, Detroit, MI). Bioassays were performed in 96-well ELISA plates, and the final volume of the preparations was 0.3 mL per well. Working solutions of exudates (2,500 and 5,000 ppm) in PDB were tested. ELISA plates were wrapped with aluminum foil and moistened filter paper and incubated at 24 °C in the dark for 24 to 48 h depending on the species. Spores suspended in PDB served as control. The whole experiment was conducted in triplicate and repeated three times. Conidial germination of at least 100 spores per replication was examined under a binocular light microscope (magnification ×600). Conidia having a germination tube longer than half of their diameter were considered as germinated.

Assay of Antibacterial Activity. A single bacterial colony of representative Gram positive and negative bacteria (**Table 1**) was picked from a 24 h old LBA culture previously prepared using the streak quadrant technique and inoculated into fresh LB broth (3 mL) in glass tubes. Cells were grown under continuous agitation (120 rpm) for 24 h at 28 or 38 °C, and their concentration was adjusted to provide an absorbance of $A_{600} = 0.01$. Absorbance was measured using a NanoDrop ND-1000 spectrophotometer.

The antibacterial activity was estimated using the agar well diffusion bioassay (*35*). Briefly, aliquots (80 μL) of bacterial cultures were added in Petri plates (9 cm in diameter) containing 15 mL of LBA and were uniformly spread with a glass rod. Filter sterilized water solutions (40 μL) of exudates (10,000 ppm) were carefully added in each well made by a cork borer (5 mm in diameter). In total, three wells were made on each plate. Control treatment consisted of depositing sterile distilled water in the wells whereas water solutions of penicillin (50 ppm) served as positive controls. Experiments were replicated three times with three replications each. All plates were incubated for 24 h at 28 or 38 °C depending on the bacterial species. Inhibition zones were used to assess the antibacterial activity of exudates.

Microtox Assay. The Microtox Analyzer model 500 (Strategic Diagnostics Inc., Newark, DE) was used for the toxicity assessment of exudates as recommended by the manufacturer for the given application. The principle behind the assay is the estimation of alterations of the natural luminescence emitted by the photobacterium *V. fischeri* in response to treatments. Decreasing values of luminescence indicate increased bioactivity of the preparations. Dry exudates were diluted in diluent, and after an initial screening, the concentration of 1,000 ppm was selected as the starting concentration for the test. Acute toxicity was expressed as the median effective concentration (EC_{50}) with end points of 5 min and 15 min. The pH of the test preparations was adjusted to 7.0, and the treatments were replicated three times.

Statistical Analysis. Statistical analyses of data were performed using the JMP software v.4.0.2 (SAS Institute, Cary, NC) applying Tukey's HSD test ($P \leq 0.05$). The homogeneity of data (significance of variance) generated from different experiments was tested performing a χ^2 test using the SPSS Statistics software v.17.0 (SPSS Inc., Chicago, IL). Values of $P \leq 0.05$ indicated that data from different experiments could be combined and analyzed as different replicates.

FT-ICR/MS Analysis of Sclerotial Exudates. *Sample Preparation*. Dry exudates (2 mg) were dissolved in 0.5 mL of a mixture of methanol:formic acid (0.1% v/v), (50:50, v/v) or methanol:ammonium hydroxide (0.1%, v/v), (50:50, v/v) for analyses in positive and negative modes, respectively. Three biological replications were performed. In order to evaluate the presence of compounds not related to the biological material (i.e., contamination during sample preparation, solvent impurities, instrument contamination) blank samples were also analyzed.

Instrumentation and Analytical Conditions. Analyses were performed in the positive and negative modes using an IonSpec Explorer FT-ICR/MS (IonSpec Inc., Lake Forest, CA) equipped with a Z-spray source (Waters Corporation, Milford, MA), a quadrupole ion guide, a standard cylindrical ion cyclotron resonance (ICR) cell, and an actively shielded superconducting magnet of 7 T. Ions were generated from an electrospray ionization (ESI) source. Samples were directly infused at a flow rate of 0.5−1.0 μL min^{-1} through a 100 μL Hamilton syringe. ESI spectra were acquired over the m/z range of 100−1,000 Da, and a total of 10 scans were collected per sample. All experimental events were controlled by the Omega8 software (IonSpec Inc.), and analyses were performed at a resolution of 100,000 fwhm. Instrument calibration was performed daily using the analytical standards recommended by the manufacturer. Raw FT-ICR/MS data were exported to MS Excel.

Data Processing and Analysis. Initially, searches for the ionization modes were performed using the metabolite-species database KNApSAcK (http://kanaya.aist-nara.ac.jp/KNApSAcK/), which contains 41,644 records of approximately 21,118 metabolites representing 42% of the total known secondary metabolites (*28*).

In a second step, based on analyses using the KNApSAcK library and identified compounds performing GC/MS analyses, putative neutral compounds were detected and all further calculations were based on the accurate masses after adduct removal. Candidate molecular forms for each peak were calculated using an elemental composition calculator (www.wsearch.com.au). For ions with a mass below 500 Da, searches were performed for the molecular forms $^{12}C_{(<39)}{}^{1}H_{(<72)}{}^{14}N_{(<20)}{}^{16}O_{(<20)}{}^{31}P_{(<9)}$-$^{32}S_{(<10)}$, whereas for ions with higher mass, searches for the $^{12}C_{(<78)}$-$^{1}H_{(<126)}{}^{14}N_{(<20)}{}^{16}O_{(<27)}{}^{31}P_{(<9)}{}^{32}S_{(<14)}$ molecular forms were performed according to Kind and Fiehn (*36*) within a mass error (Δ ppm) of less than 1.5 ppm. The "seven golden rules" (*36*) were applied in order to reduce the number of candidate chemical formulas for a given mass. Furthermore, using the Molecular Weight Calculator v.1.0 (www.wsearch.com.au), ions corresponding to isotopic forms of the identified molecular formula were detected providing additional information for the molecular form of the detected ions.

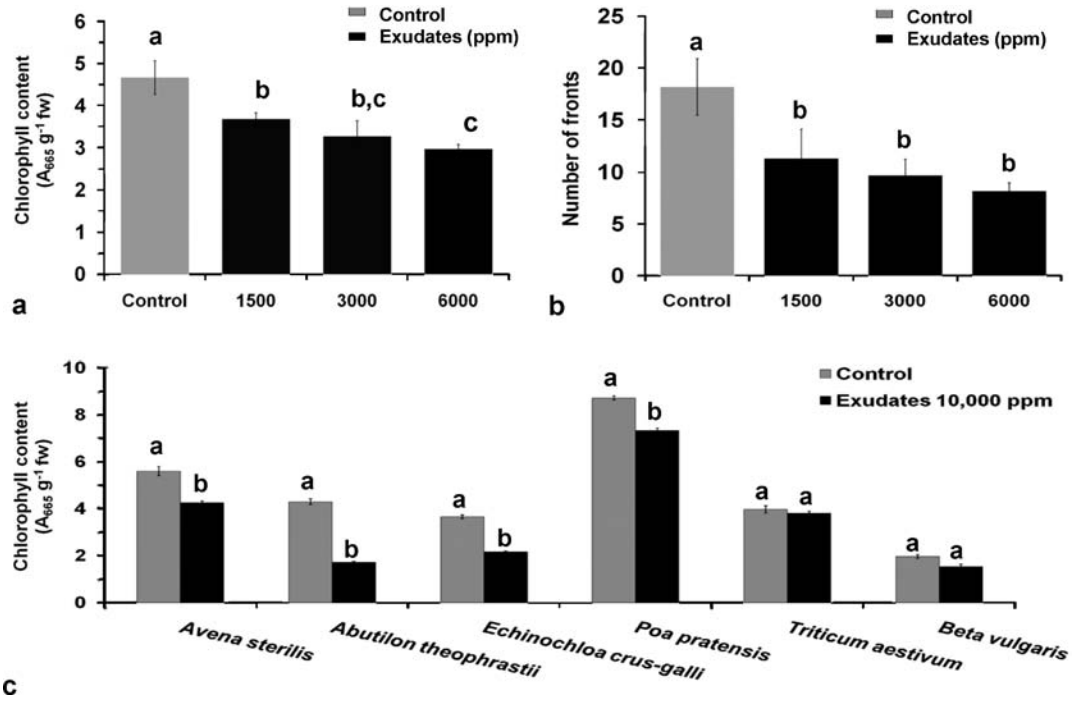

Figure 1. Effect of sclerotial exudates of *Rhizoctonia solani* AG2-2 IIIB isolate on (**a**) chlorophyll content and (**b**) number of fronds of *Lemna minor* L., and (**c**) the chlorophyll content of several plant species. Bars represent the means of three experiments with three replications each. Standard deviations are plotted as vertical bars, and means followed by the same letter are not significantly different (Tukey's HSD test, $P \leq 0.05$).

Additionally, an in-house-built target library composed of 150 compounds reported to be present in sclerotial exudates and sclerotia of several fungi was created in MS Excel. For the construction of the in-house library, data were acquired from publicly available databases [Chemspider (http://www.chemspider.com/PropertiesSearch.aspx), Kyoto Encyclopedia of Genes and Genomes LIGAND (http://www.genome.jp/kegg/ligand.html), PubChem (http://pubchem.ncbi.nlm.nih.gov/), Metlin (http://metlin.scripps.edu/metabo_search.php), and Madison Metabolomics Consortium Database (http://mmcd.nmrfam.wisc.edu/)].

GC/MS Analysis of Sclerotial Exudates. *Sample Preparation*. GC/MS analyses were performed complementary to FT-ICR/MS analyses. Derivatization was performed by adding 80 μL of methoxylamine hydrochloride solution (20 mg mL^{-1} in pyridine) at 30 °C for 120 min in 5 mg of dry exudates followed by the addition of 80 μL of MSTFA at 37 °C for 90 min. Blank samples were also analyzed in order to detect the presence of compounds not related to the biological material (e.g., column bleeding, contamination during sample preparation, solvent impurities).

Instrumentation and Analytical Conditions. A Varian Saturn 2100T GC/MS/MS system equipped with a CP-8400 autosampler was used for the analyses. The ionization mode was electron ionization at 70 eV. The temperature for the ion source and the transfer line was set to 150 and 250 °C accordingly. Electron impact mass spectra were recorded at a mass range of 40−650 Da at 1 scan s^{-1} rate with a 10.0 min solvent delay. A Varian CP-Sil 8 CB capillary column (30 m, i.d. 0.25 μm) was used with helium flow of 1 mL min^{-1}. Samples (1 μL) were injected using the standard on column mode with a split ratio of 10 at an injector temperature of 230 °C. The initial temperature of the oven was set at 70 °C, stable for 5 min, followed by a 5 °C min^{-1} increase to 300 °C, stable for 1 min. Three biological replications were performed.

Data Processing and Analysis. For chromatogram acquisition and peak deconvolution the Saturn GC/MS Workstation (v.5.51, Varian) was used. Library searches were performed using the NIST08 libraries (National Institute of Standards and Technology, Gaithersburg, MD) and online databases [The Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_sspq.html) and the Spectral Database for Organic Compounds SDBS (http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi)]. After the initial analysis of spectra, selected substances were verified with commercially purchased analytical standards, whereas in cases in which a very good fit could be achieved (>90%) tentative identification was performed.

^{1}H NMR Spectroscopy Analysis of Sclerotial Exudates. *Sample Preparation*. Exudates (1.0 mL) were lyophilized for 24 h and were dissolved in D_2O (1.0 mL). Solutions were centrifuged (11000*g*) for 1 h and the supernatants were subjected to a second centrifugation (11000*g*) for 30 min. Samples were prepared from the supernatants.

Instrumentation and Analytical Conditions. ^{1}H NMR spectra were recorded using a Varian Inova 500 MHz NMR spectrometer (Varian, Palo Alto, CA) equipped with a ^{1}H{^{13}C,^{15}N} triple resonance cold probe. A total of 256 transients of 32K data points were acquired per sample with a 90° pulse angle, 2 s acquisition time, and 2 s recycle delay with presaturation of H_2O during the recycle delay.

Data Processing and Analysis. Spectra were processed and analyzed as described previously (*32*). Baseline was automatically corrected, and offsets of chemical shifts were corrected based on the reference signal of TSP (0.00 ppm) using the MesteC software v.461 (Mestrelab Research, Santiago de Compostela, Spain). Assignment of shifts to corresponding metabolites was performed using the ACD/C+H NMR Predictor and Database v.12.01 (Advanced Chemistry Development, Inc., ACD/Laboratories, Toronto, Canada) and data from the Human Metabolome Database (http://www.hmdb.ca).

RESULTS

Production of Exudates and Physicochemical Properties. Exudates are produced in *R. solani* AG2-2 IIIB cultures two weeks after the initial inoculation of the media, as yellowish droplets turning rapidly to dark brown, in the areas of the sclerotia formation (Supplementary Figure 1 in the Supporting Information). Their production is continuous for up to eight weeks under the described conditions (data not shown), and approximately 150 mL of exudates could be collected from 100 Petri plates. They are freely soluble in water, and the pH of their water solutions is slightly basic, close to 7.5, in contrast to the more acidic exudates of *S. rolfsii* (pH 4.5) and *Sclerotinia sclerotiorum* (pH 5.4) (*37*, *38*).

Phytotoxicity of Exudates to *Lemna minor* and Terrestrial Plants. All throughout the experiments, untreated *L. minor* and young seedlings of various crop species exhibited no signs of stress (data not shown). Compared to the control treatments, all

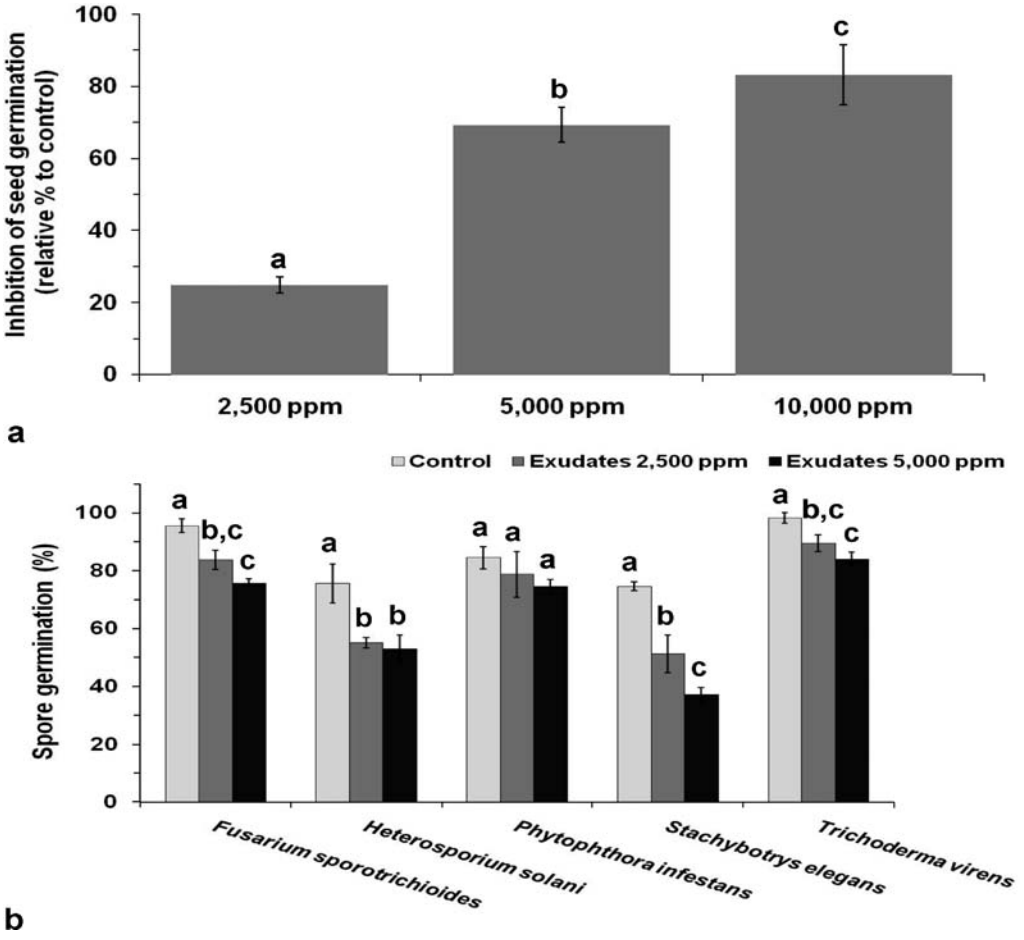

Figure 2. Effects of sclerotial exudates of *Rhizoctonia solani* AG2-2 IIIB isolate on (**a**) the germination of *Lactuca sativa* L. seeds 48 h after treatments, and (**b**) the spore germination of several fungal species. Bars represent the means of three experiments with three replications each. Standard deviations are plotted as vertical bars, and means followed by the same letter are not significantly different (Tukey's HSD test, $P \leq 0.05$).

working doses of the sclerotial exudates significantly decreased the chlorophyll content of *L. minor* (**Figure 1a**). Treatment with exudates at 6000 ppm reduced the number of fronds by 55.1% (**Figure 1b**).

At 10,000 ppm, exudates had a varied and significant effect on the chlorophyll content of four out of six plant species (**Figure 1c**). Affected seedlings had their leaves discolored. Compared to the control, there was a significant decrease in the chlorophyll content of treated *A. theophrastii* (60.23%) followed by *E. crus-galli* (40.66%), *A. sterilis* (24.11%), and *P. pratensis* (15.94%). The remaining two crop species, *B. vulgaris* and *T. aestivum*, were not affected. Additionally, relative to the control, percent germination of *L. sativa* seeds significantly decreased with an IC_{50} of 4,522 ppm 48 h after treatment (**Figure 2a**).

Antifungal and Antibacterial Activities of Exudates. Spore germination of all fungi except for *P. infestans* was affected by all doses of *R. solani* exudates (**Figure 2b**). The sensitivity of different fungi varied considerably compared to the control (Tukey's HSD test, $P \leq 0.05$). At the concentration of 5,000 ppm, exudates significantly reduced spore germination of *S. elegans* by 50.25%, followed by *H. solani* (29.83%), *F. sporotrichioides* (29.83%), and *T. virens* (14.48%) relative to the control (**Figure 2b**). Sclerotial exudates applied at the maximum applied concentration of 10,000 ppm had no effect on the growth of bacteria (data not shown).

Toxicological Assessment of Exudates Using Microtox. The acute 5 and 10 min acute EC_{50} values of exudates to the bioluminescent bacterium *V. fischeri* were 354.9 and 365.5 ppm, respectively (**Figure 3**). These results suggest that the effect of exudates on the bacterium was almost completed within the first 5 min.

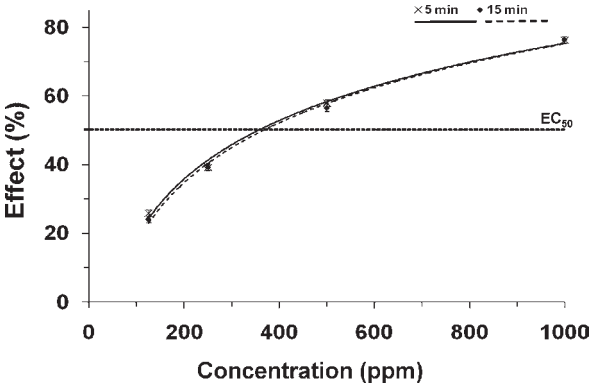

Figure 3. Effect of sclerotial exudates of *Rhizoctonia solani* AG2-2 IIIB isolate on the luminescence of the photobacterium *Vibrio fischeri* expressed as median effective concentration (EC_{50}) with end points of 5 min and 15 min using the Microtox assay. Each data point represents the means of three experiments with three replications each. Standard deviations are plotted as vertical bars, and means followed by the same letter are not significantly different (Tukey's HSD test, $P \leq 0.05$).

Chemical Composition of Exudates. FT-ICR/MS (**Figure 4**) analyses of *R. solani* sclerotial exudates revealed the complexity of their chemical composition. Integrating data obtained from

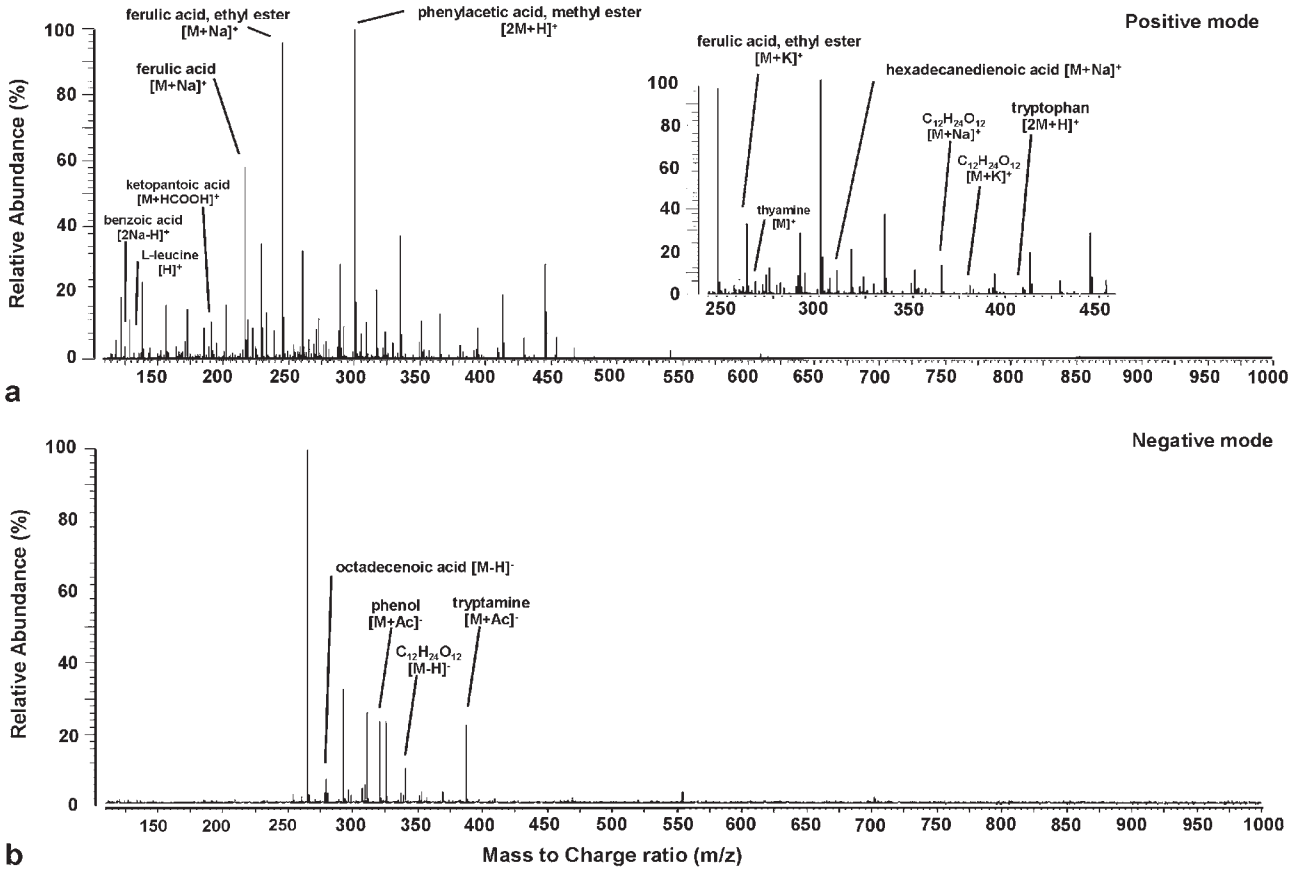

Figure 4. Representative FT-ICR/MS spectra of sclerotial exudates of *Rhizoctonia solani* AG2-2 IIIB isolate acquired in positive (**a**) and negative (**b**) modes in the mass range 100−1,000 Da. Annotations of representative metabolites are displayed.

FT-ICR/MS (**Figure 4**), GC/MS (**Figure 5**), ^{1}H NMR (**Figure 6**), and the KNApSAcK and in-house-built libraries, more than 90 compounds were identified (**Tables 2** and **3**). The KNApSAcK library provides an automated search for the positive {$[M - e]^+$, $[M + H]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M + K]^+$} and negative {$[M + e]^-$, $[M - H]^-$} modes. In addition, with appropriate modifications in the data set, searches for $[2M + H]^+$, $[M + 2Na - H]^+$ $[M + HCOOH + H]^+$, $[M + {}^{39}K - H]^+$, $[M + {}^{41}K]^+$, $[M + Na - 2H]^-$, $[M + {}^{35}Cl]^-$, $[M + {}^{37}K]^-$, $[M + Ac]^-$, and $[M + {}^{39}K - 2H]^-$ ionization modes were also performed.

FT-ICR/MS Analysis. Identified metabolites represent more than 60% and 15% of the total amplitude of spectra in the positive and negative modes, respectively (**Tables 2** and **3**). Analyses in the positive mode enabled the detection of neural ions within an average error of less than 1 ppm under several ionization modes, with protonated and adducts of Na^+ to be the most abundant ones. Among the identified compounds, phenolics (17.40%), carboxylic acids (12.79%), carbohydrates (6.08%), fatty acids (3.78%), and amino acids (3.47%) were the predominant chemical groups (**Figure 7a**). In contrast, analyses in the negative mode revealed the presence of a small number of compounds in low concentrations as indicated by the low total amplitude of spectra (data not shown). Detected ions were assigned to compounds belonging to alkaloids (1.61%), carbohydrates (1.61%), carbolic acids (8.44%), fatty acids (0.91%) and heterocyclic compounds (0.48%) (**Table 3**, **Figure 7b**).

GC/MS Analysis. Analyses of exudates performing GC/MS confirmed the results of FT-ICR/MS and revealed the presence of amino, carboxylic and lipid acids, and several carbohydrates (**Tables 2** and **3**, **Figure 5**). Nonetheless, the number of detected metabolites applying GC/MS was significantly smaller than those

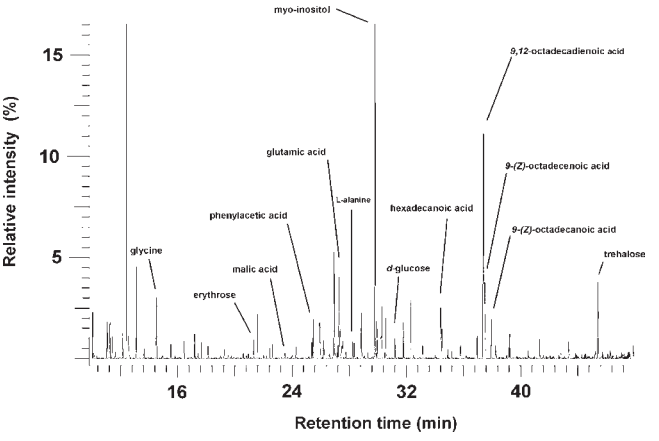

Figure 5. Representative GC/MS total-ion chromatogram of sclerotial exudates of *Rhizoctonia solani* AG2-2 IIIB isolate. Annotations of representative metabolites are displayed.

detected by FT-ICR/MS, which further supports the choice of the latter as the principal analytical platform for chemical analyses of exudates.

^{1}H NMR Spectroscopy. ^{1}H NMR spectroscopy qualitatively confirmed results of FT-ICR/MS and GC/MS analyses. Because of the complexity of the composition of exudates, a large number of overlapping peaks could be detected in ^{1}H NMR spectra, especially in the area of carbohydrates (shifts 3−6 ppm) (**Figure 6**), making the assignment of peaks to corresponding metabolites difficult. Such presence of a large number of carbohydrates in the NMR spectra confirms findings of FT-ICR/MS

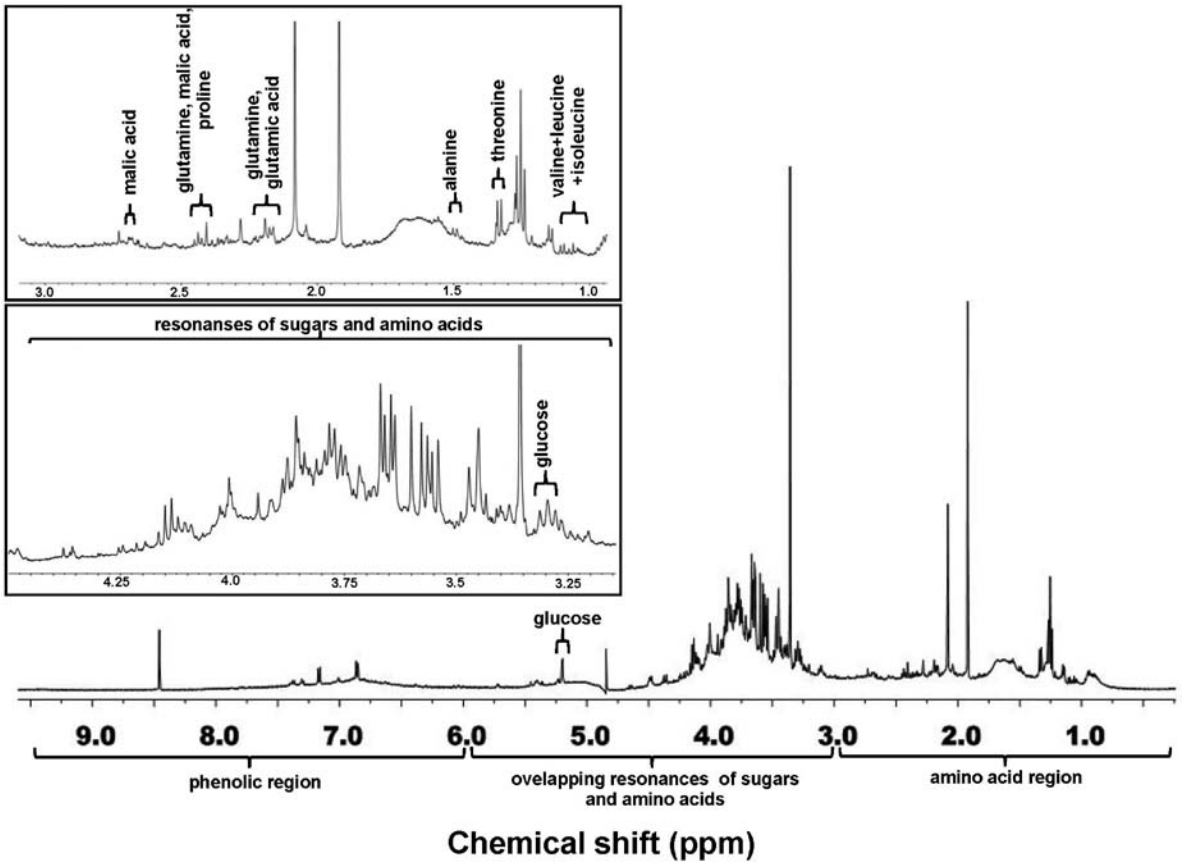

Figure 6. Representative ^{1}H NMR spectrum of sclerotial exudates *Rhizoctonia solani* AG2-2 IIIB isolate. Annotations of representative metabolites and regions are displayed are displayed.

and GC/MS analyses (**Tables 2** and **3**). A large number of peaks were also recorded in the area of amino acids (shifts 0.5−3 ppm). Among those, shifts were assigned to alanine, isoleucine, glucose, glutamic acid, glutamine, leucine, malic acid, and threonine (**Figure 6**). Furthermore, the presence of shifts in the area between 6 and 9 ppm is indicative of phenolics present in the exudates.

DISCUSSION

As far as we are aware, the present study is the first integrated approach using different analytical platforms for the deconvolution of the metabolic composition of *R. solani* sclerotial exudates and the assessment of their bioactivity and toxicity.

Metabolic Composition of Exudates. FT-ICR/MS, GC/MS, and ^{1}H NMR analyses revealed that *R. solani* AG2-2 IIIB sclerotial exudates are complex mixtures composed mainly of phenolics, carboxylic acids, carbohydrates, and fatty acids. The applied analytical protocols enabled a more comprehensive deconvolution of their metabolic composition compared to previous studies where analysis of fungal sclerotial exudates was based on analytical methodologies with limited capabilities (*10*, *21*, *22*, *37*, *39*).

Phytotoxic and Antifungal Acitvities of Exudates. PAA and its methyl ester are the most abundant metabolites of the carboxylic fraction of exudates. PAA is a well studied phytotoxic compound of *R. solani* (*14*−*16*) and has been implicated in pathogenesis and promotion of Rhizoctonia disease syndrome in potato in the absence of the pathogen (*40*). Another metabolite detected in the exudates is the aromatic amino acid phenylalanine, which is a product of the shikimic acid pathway, and is considered a precursor of PAA (*41*, *42*). The correlation of phenylalanine with the degree of pathogenicity of *R. solani* virulent strains of potato has been established (*43*). Taking together the above findings, it is reasonable to assume that *R. solani* AG2-2 IIIB sclerotial exudates play a role in pathogenesis.

Based on FT-ICR/MS analyses in positive mode, phenolics are the major component of exudates with ferulic acid and its ethyl ester to be the most abundant. Many of these phenolics have been reported to exhibit phytotoxic (*44*−*46*), antioxidant (*47*), and/or fungitoxic activities (*10*, *48*, *49*). Pandey et al. (*10*) correlated the presence of ferulic and chlorogenic acids in sclerotial exudates of *S. rolfsii* to their fungitoxicity. Thus, the observed inhibition of fungal spore germination in the present investigation can be partially attributed to the presence of phenolics.

The antimicrobial activities of fatty acids and their derivatives are well-known, and their mode of action is extensively studied (*50*). Among the fatty acids detected in exudates, dodecanoic (syn. lauric), tetradecanoic (syn. myristic), hexadecanoic (syn. palmitic), and octadecenoic (syn. oleic) acids have been reported to be active against important fungal plant pathogens and food spoilers (*51*−*54*). Interestingly, hexadecanoic, octadecenoic, tetradecanoic, heptadecanoic (syn. margaric), and octadecanoic (syn. stearic) acids are known to have antimycotic activity against the dermatophytes *Trichophyton* sp. and *Microsporum canis* (*55*). In addition to lipid acids, two lipid hydroperoxides identified in exudates, namely, hydroperoxy-octadecadienoic and hydroperoxy-octadecatrienoic acids, are well established as antifungal compounds (*56*). Also, several identified benzoic acid derivatives and salicylic acid exhibit antifungal activity against several plant pathogens (*57*). The fact that the sclerotial exudates contain a range of fatty acids, some of which are antifungal against economically important plant pathogens, food spoilers or dermatophytes, makes them a rich source of bioactive compounds.

Table 2. Chemical Composition of Sclerotial Exudates of *Rhizoctonia solani* AG2-2 IIIB Isolate Based on Identified Metabolites Performing FT-ICR/MS Analyses in the Positive Mode[a]

metabolite	molecular formula	monoisotopic mass	av absolute mass error (ppm)	ion form	rel composition ± SD[b] (%)
			Amines		
histamine	$C_5H_9N_3$	111.0796	0.6	$[M + HCOOH + H]^+$	0.174 ± 0.032
phenethylamine (alkaloid)	$C_8H_{11}N$	121.0891	1.3	$[M + 2Na - H]^+$	0.143 ± 0.019
tyramine	$C_8H_{11}NO$	137.0841	1.6	$[2M + H]^+$	0.070 ± 0.015
			Amino Acids		
L-alanine[c]	$C_3H_7NO_2$	89.0477	0.5	$[2M + H]^+$	0.056 ± 0.017
L-arginine	$C_6H_{14}N_4O_2$	174.1117	1.2	$[M + K]^+$, $[2M + H]^+$	0.112 ± 0.034
L-asparagine[c]	$C_4H_8N_2O_3$	132.0535	0.01	$[M + Na]^+$, $[M + NH_4]^+$, $[M + HCOOH + H]^+$	0.243 ± 0.052
cystathionine	$C_7H_{14}N_2O_4S$	222.0674	1.6	$[M + HCOOH + H]^+$	0.389 ± 0.104
glutamic acid[c]	$C_5H_9NO_4$	147.0532	1.5	$[M + NH_4]^+$	0.085 ± 0.022
glutamine[c]	$C_5H_{10}N_2O_3$	146.0691	0.06	$[M + K]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M + 2Na - H]^+$, $[M + HCOOH + H]^+$	0.446 ± 0.175
glycine[c]	$C_2H_5NO_2$	75.0320	1.7	$[2K^{39} - H]^+$	0.086 ± 0.021
histidine	$C_6H_9N_3O_2$	155.0695	1.5	$[M]^+$, $[M + H]^+$	0.163 ± 0.028
L-leucine/L-isoleucine	$C_6H_{13}NO_2$	131.0946	0.04	$[M + H]^+$, $[M + Na]^+$	0.222 ± 0.054
lysine	$C_6H_{14}N_2O_2$	146.1055	0.8	$[M + HCOOH + H]^+$	0.074 ± 0.023
methionine	$C_5H_{11}NO_2S$	149.0510	0.08	$[2M + H]^+$	0.182 ± 0.041
pantothenic acid	$C_9H_{17}NO_5$	219.1107	1.4	$[M + NH_4]^+$	0.064 ± 0.019
phenylalanine	$C_9H_{11}NO_2$	165.0790	1.8	$[M]^+$	0.076 ± 0.021
L-proline[c]	$C_5H_9NO_2$	115.0633	0.1	$[M + H]^+$, $[M + Na]^+$, $[M + K]^+$	0.342 ± 0.112
L-serine	$C_3H_7NO_3$	105.0426	1	$[2M + H]^+$	0.074 ± 0.017
threonine	$C_4H_9NO_3$	119.0582	1.7	$[M + HCOOH + H]^+$	0.303 ± 0.087
tryptophan	$C_{11}H_{12}N_2O_2$	204.0899	1.9	$[M + HCOOH + H]^+$, $[2M + H]^+$	0.359 ± 0.131
L-valine[c]	$C_5H_{11}NO_2$	117.0790	0.07	$[M + H]^+$, $[M + Na]^+$	0.190 ± 0.023
			Carboxylic Acids and Esters		
methylbutyl-succinic acid, methyl ester	$C_{11}H_{20}O_4$	216.1362	0.5	$[M + Na]^+$	0.086 ± 0.024
benzoic acid	$C_7H_6O_2$	122.0368	0.09	$[M + Na]^+$, $[M + NH_4]^+$	0.328 ± 0.076
dihydrodipicolinic acid	$C_7H_7NO_4$	169.0375	1.4	$[M + NH_4]^+$	0.104 ± 0.031
ethyl gallate	$C_9H_{10}O_5$	198.0528	1.4	$[M + HCOOH + H]^+$	0.155 ± 0.019
gallic acid	$C_7H_6O_5$	170.0215	1.4	$[M + HCOOH + H]^+$	0.285 ± 0.041
glyceric acid	$C_3H_6O_4$	106.0266	0.5	$[M + 2Na - H]^+$	0.044 ± 0.008
ketopantoic acid	$C_6H_{10}O_4$	146.0579	0.03	$[M + Na]^+$, $[M + K]^+$, $[M + 2Na - H]^+$, $[M + HCOOH + H]^+$	0.447 ± 0.132
2-keto-3-methylvaleric acid	$C_6H_{10}O_3$	130.0630	0.2	$[M + Na]^+$, $2M + K]^+$, $[2M + H]^+$	1.226 ± 0.432
malic acid[c]	$C_4H_6O_5$	134.0215	1.7	$[M + HCOOH + H]^+$	0.049 ± 0.011
methyl gallate	$C_8H_8O_5$	184.0372	1.6	$[M + H]^+$	0.075 ± 0.017
methylcinnamate	$C_{10}H_9O_2$	161.0608	1.6	$[M + H]^+$	0.067 ± 0.013
2-methoxybenzoic acid	$C_8H_8O_3$	152.0473	1.6	$[M + H]^+$	0.212 ± 0.022
mevalonic acid	$C_6H_{12}O_4$	148.0736	0.07	$[M + Na]^+$	0.175 ± 0.023
phenylacetic acid, methyl ester[c]	$C_9H_{10}O_2$	150.0681	0.4	$[M + Na]^+$, $[2M + H]^+$	8.888 ± 2.103
phenylacetic acid[c]	$C_8H_8O_2$	136.0524	1.3	$[M + H]^+$, $[M + NH_4]^+$, $[2M + H]^+$, $[M + HCOOH + H]^+$	0.129 ± 0.018
phloretic acid	$C_9H_{10}O_3$	166.0630	1.3	$[M]^+$, $[M + Na]^+$	0.208 ± 0.031
1-pyrroline-5-carboxylate[c]	$C_5H_7NO_2$	113.0477	1.4	$[M + H]^+$	0.049 ± 0.013
salicylic acid	$C_7H_6O_3$	138.0317	1.6	$[M + H]^+$	0.105 ± 0.015
valeric acid[c]	$C_5H_{10}O_2$	102.0681	0.1	$[M + Na]^+$	0.071 ± 0.024
			Fatty Acids		
12-hydroxydodecanoic acid	$C_{12}H_{24}O_3$	216.1725	0.03	$[M + Na]^+$, $[M + K]^+$	0.880 ± 0.111
octanoic acid	$C_8H_{16}O_2$	144.1150	1.3	$[M + K^{41}]^+$	0.075 ± 0.021
dodecandioic acid[c]	$C_{12}H_{22}O_4$	230.1518	0.5	$[M + Na]^+$	0.076 ± 0.017
epoxy octadecanoic acid	$C_{18}H_{34}O_3$	298.2508	0.1	$[M + Na]^+$	0.077 ± 0.012
heptadecanoic acid	$C_{17}H_{34}O_2$	270.2559	0.5	$[M + NH_4]^+$	n/a[d]
hexadecanedioic acid	$C_{16}H_{30}O_4$	286.2144	0.06	$[M + Na]^+$, $[M + K]^+$	1.090 ± 0.206
hydroperoxy-octadecadienoic acid	$C_{18}H_{32}O_4$	312.2301	0.1	$[M + Na]^+$	0.166 ± 0.023
hydroperoxy-octadecatrienoic acid	$C_{18}H_{30}O_4$	310.2144	0.03	$[M + Na]^+$	0.145 ± 0.031
dodecanoic acid (syn. lauric acid)	$C_{12}H_{24}O_2$	200.1776	1.6	$[M + K^{41}]^+$	0.128 ± 0.017
tetradecanoic acid	$C_{14}H_{28}O_2$	228.2089	0.03	$[M + Na]^+$, $[M + K]^+$	0.133 ± 0.029
hexadecanoic acid	$C_{16}H_{32}O_2$	256.2402	0.09	$[M + NH_4]^+$	n/a
hexadecenoic acid[c]	$C_{16}H_{30}O_2$	254.2246	0.1	$[M + Na]^+$	0.113 ± 0.021
octadecanoic acid[c]	$C_{18}H_{36}O_2$	284.2715	0.1	$[M + NH_4]^+$	0.072 ± 0.025
trihydroxy-octadecadienoic acid	$C_{18}H_{32}O_5$	328.2250	0.5	$[M + Na]^+$	0.097 ± 0.028

Table 2. Continued

metabolite	molecular formula	monoisotopic mass	av absolute mass error (ppm)	ion form	rel composition ± SD[b] (%)
Carbohydrates and Esters					
Monosaccharides					
D-glucose[c]	$C_6H_{12}O_6$	180.0634	0.01	$[M + Na]^+$, $[M + K]^+$, $[M + 2Na - H]^+$	n/a
deoxyribose[c]	$C_5H_{10}O_4$	134.0579	0.6	$[M + Na]^+$, $[M + K]^+$	0.24 ± 0.077
fructose[c]	$C_6H_{12}O_6$	180.0634	0.01	$[M + Na]^+$, $[M + K]^+$, $[M + 2Na - H]^+$	n/a
mannitol-1-phosphate	$C_6H_{15}O_9P$	262.0454	1.6	$[M + H]^+$	0.169 ± 0.028
glucosamine 6-phosphate	$C_6H_{14}NO_8P$	259.0457	0.02	$[M + H]^+$, $[M + NH_4]^+$	0.161 ± 0.035
fructose-6-phosphate	$C_6H_{13}O_9P$	260.0297	0.6	$[M + H]^+$	0.167 ± 0.030
Polysaccharides					
trehalose[c]	$C_{12}H_{22}O_{11}$	342.1162	0.17	$[M + Na]^+$, $[M + K]^+$	n/a
sucrose[c]	$C_{12}H_{22}O_{11}$	342.1162	0.17	$[M + Na]^+$, $[M + K]^+$	n/a
maltose[c]	$C_{12}H_{22}O_{12}$	342.1162	0.17	$[M + Na]^+$, $[M + K]^+$	n/a
Sugar Alcohols					
erythritol[c]	$C_4H_{10}O_4$	122.0579	1.9	$[2M + H]^+$	0.800 ± 0.203
glycerol	$C_3H_8O_3$	92.0473	0.16	$[M + H]^+$, $[M + Na]^+$	0.190 ± 0.026
mannitol[c]	$C_6H_{14}O_6$	182.0790	0.05	$[M + Na]^+$, $[2M + H]^+$	0.382 ± 0.097
myo-inositol[c]	$C_6H_{12}O_6$	180.0634	0.01	$[M + Na]^+$, $[M + K]^+$, $[M + 2Na - H]^+$	n/a
glycerol 3-phosphate	$C_3H_9O_6P$	172.0137	0.05	$[M + H]^+$	0.051 ± 0.011
Tetrose Carbohydrates					
erythrose [c]	$C_4H_8O_4$	120.0423	0.2	$[M + Na]^+$	0.040 ± 0.013
Glycosylamines					
adenosine	$C_{10}H_{13}N_5O_4$	267.0968	0.3	$[M + NH_4]^+$	0.052 ± 0.020
deoxyadenosine	$C_{10}H_{13}N_5O_3$	251.1018	0.3	$[M + NH_4]^+$	0.169 ± 0.031
Heterocyclic Compounds					
2,4-dimethylquinazoline	$C_{10}H_{10}N_2$	158.0844	1.6	$[M]^+$	0.080 ± 0.023
dihydrozeatin	$C_{10}H_{15}N_5O$	221.1277	1.4	$[M + K]^+$, $[2M + H]^+$, $[M + NH_4]^+$	0.126 ± 0.031
ellagic acid	$C_{14}H_6O_8$	302.0063	1.4	$[M + H]^+$	0.130 ± 0.057
guanine	$C_5H_5N_5O$	151.0494	0.3	$[M + NH_4]^+$	0.093 ± 0.029
quinoline	C_9H_7N	129.0579	0.1	$[M + Na]^+$, $[2M + H]^+$	0.330 ± 0.120
thiamine	$C_{12}H_{17}N_4OS$	265.1118	1.6	$[M]^+$	0.460 ± 0.124
Phenolics and Esters					
chlorogenic acid	$C_{16}H_{18}O_9$	354.0951	0.2	$[M + Na]^+$	0.055 ± 0.011
coumaric acid	$C_9H_8O_3$	164.0473	1.8	$[M + NH_4]^+$	0.051 ± 0.012
ferulic acid	$C_{10}H_{10}O_4$	194.0579	0.01	$[M + H]^+$, $[M + K]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M + HCOOH + H]^+$	5.992 ± 1.534
ferulic acid, ethyl ester	$C_{12}H_{14}O_4$	222.0892	0.08	$[M + H]^+$, $[M + Na]^+$	11.29 ± 1.433
protocatechuic acid	$C_7H_6O_4$	154.0266	0.2	$[M]^+$	0.060 ± 0.014
Ureas					
1,3-diphenethylurea	$C_{17}H_{20}N_2O$	268.1576	1.5	$[M + H]^+$	0.052 ± 0.019
phenylthiourea	$C_7H_8N_2S$	152.0408	1.2	$[M + Na]^+$	0.061 ± 0.014
Other Organic Compounds					
2-acetolactate	$C_5H_7O_4$	131.0350	1.8	$[M]^+$, $[M + H]^+$	0.145 ± 0.029
ascorbic acid	$C_6H_8O_6$	176.0321	1.8	$[M + NH_4]^+$	0.177 ± 0.037
dodecanol	$C_{12}H_{26}O$	186.1984	1.6	$[M + K]^+$	0.089 ± 0.021
pyridoxamine phosphate	$C_8H_{13}N_2O_5P$	248.0562	1.7	$[M + HCOOH + H]^+$	0.057 ± 0.014

[a] Data were retrieved from the databases Chemspider, KEGG-LIGAND, PubChem, Metlin, and Madison Metabolomics Consortium Database. [b] The means of three biological replications with standard deviations (SD) are displayed. [c] Compounds detected in both FT-ICR/MS and GC/MS analyses. [d] Compounds with identical monoisotopic masses for which the relative composition cannot be determined applying direct infusion FT-ICR/MS.

Toxicological Risk Assessment of Exudates. The results of the toxicological risk assessment showed that *R. solani* sclerotial exudates are bioactive with moderate values of EC_{50} that are comparable to those of commercially developed herbicides such as diuron, glyphosate, mesotrione, norflurazon, oxadiazon, and paraquat (*58*). This finding holds promise as an alternative to fungal-based pesticides whose toxicity to nontarget organisms and relative slow effectiveness compared to chemical pesticides have hindered their development and use (*59*).

Role of Exudates in Fungal Physiology. Formation of sclerotial exudates is a phenomenon that is linked, at least in the case of *Sclerotinia* and *Sclerotiorum* species, to cell death and the rupture

Table 3. Chemical Composition of Sclerotial Exudates of *Rhizoctonia solani* AG2-2 IIIB Isolate Based on Identified Metabolites Performing FT-ICR/MS Analyses in the Negative Mode[a]

metabolite	molecular formula	monoisotopic mass	av absolute mass error (ppm)	ion form	rel composition ± SD[b](%)
		Alkaloids			
tryptamine	$C_{10}H_{12}N_2$	160.1000	1.9	$[M + Ac]^-$	5.986 ± 1.458
		Carbohydrates			
trehalose[c]	$C_{12}H_{22}O_{11}$	342.1162	1.1	$[M - H]^-$	n/a[d]
sucrose[c]	$C_{12}H_{22}O_{11}$	342.1162	1.1	$[M - H]^-$	n/a
maltose[c]	$C_{12}H_{22}O_{11}$	342.1162	1.1	$[M - H]^-$	n/a
		Carbolic Acids			
phenol	C_6H_6O	94.0419	1.8	$[M + Ac]^-$	8.439 ± 1.287
		Heterocyclic Compounds			
adenine	$C_5H_5N_5$	135.0545	1.7	$[M - H]^-$	0.476 ± 0.214
		Fatty Acids and Esters			
octadecenoic acid[c]	$C_{18}H_{34}O_2$	282.2559	0.5	$[M - H]^-$	0.906 ± 0.156

[a] Data were retrieved from the databases Chemspider, KEGG-LIGAND, PubChem, Metlin, and Madison Metabolomics Consortium Database. [b] The means of three biological replications with standard deviations (SD) are displayed. [c] Compounds detected in both FT-ICR/MS and GC/MS analyses. [d] Compounds with identical monoisotopic masses for which the relative composition cannot be determined applying direct infusion FT-ICR/MS.

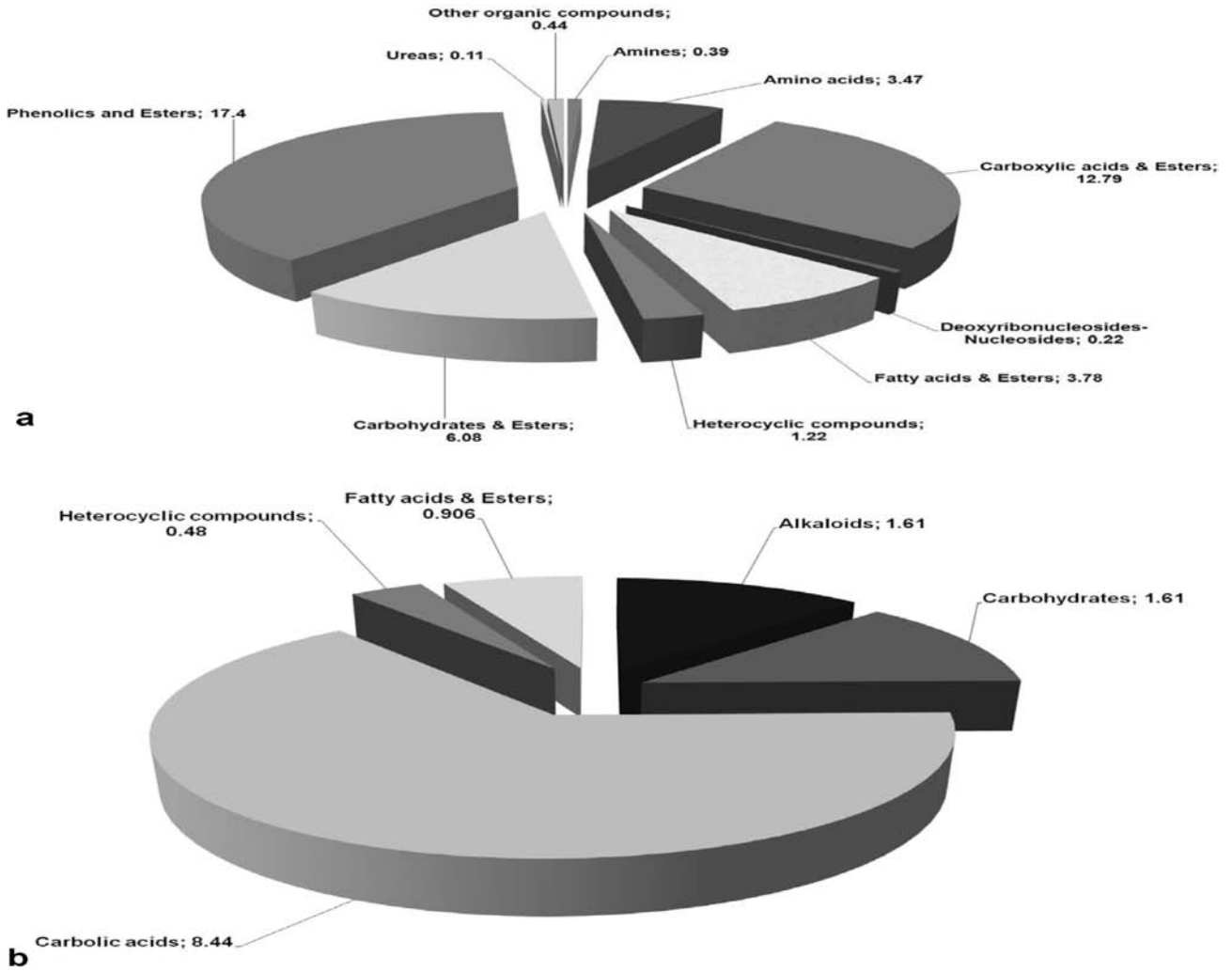

Figure 7. Relative composition (%) of sclerotial exudates of *Rhizoctonia solani* AG2-2 IIIB isolate applying FT-ICR/MS analyses in the positive (**a**) and negative (**b**) modes.

of cytoplasmic membrane in the developing sclerotia and/or translocation of excess nutrients, water, and metabolites to mature sclerotia in order to sustain the increased metabolic activity (*38*, *60*, *61*). In the case of *R. solani* sclerotia, a progressive

increase in the concentration of certain metabolites such as trehalose and several fatty and carboxylic acids with age was recently confirmed (*32*). These metabolites play an important role in fungal physiology (*46*, *62*−*65*). The similarity of the metabolic composition of the sclerotia to that of the exudates highly suggests that they are of cytoplasmic or membranous origin.

The deconvolution of metabolic composition of exudates also revealed the presence of fatty acid hydroperoxides and the antioxidant compounds, ascorbic and protocatechuic acids, as well as phenolics that are reportedly known to be involved in oxidative processes triggered by reactive oxygen species (ROS) in biological systems (*43*, *47*). The presence of such compounds is indicative of a physiological role of exudates in the ROS-mediated differentiation processes of *R. solani* sclerotia. These findings are in support of the reported involvement of oxidative processes in sclerotial differentiation in several fungi including *R. solani* (*38*). The above findings support the notion that metabolites present in the exudates are highly unlikely excreted to be wasted, but rather they seem to be products of cell destruction and have multiple physiological roles (*66*).

In conclusion, the sclerotial exudates of *R. solani* AG2-2 IIIB represent a potential bioactive source with moderate toxicity whose chemical composition could be used as template for the synthesis of bioactive mixtures of chemicals. Furthermore, we have shown with certainty that the integration of different analytical platforms is a powerful approach for extracting the most and reliable information on the metabolic composition of complex biological samples.

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Supporting Information Available: Figure depicting a photo of three-week old *Rhizoctonia solani* AG2-2 IIIB sclerotia producing dark brown liquid exudates. This material is available free of charge via the Internet at http://pubs.acs.org.

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