Metabolic Profiling of Root Exudates of *Arabidopsis thaliana*

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This paper was withdrawn on October 7, 2009 (*J. Agric. Food Chem.* **2009**, *57*, 9346).

In addition to accumulating biologically active chemicals, plant roots continuously produce and secrete compounds into their immediate rhizosphere. However, the mechanisms that drive and regulate root secretion of secondary metabolites are not fully understood. To enlighten two neglected areas of root biology, root secretion and secondary metabolism, an in vitro system implementing root-specific elicitation over a 48-day time course was developed. After roots of *Arabidopsis thaliana* had been elicited with salicylic acid, jasmonic acid, chitosan, and two fungal cell wall elicitors, the secondary metabolites subsequently secreted were profiled. High-performance liquid chromatography was used to metabolically profile compounds in the root exudates, and 289 possible secondary metabolites were quantified. The chemical structures of 10 compounds were further characterized by ^{1}H and ^{13}C NMR: butanoic acid, *trans*-cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzamide, methyl *p*-hydroxybenzoate, 3-indolepropanoic acid, syringic acid, and vanillic acid. Several of these compounds exhibited a wide range of antimicrobial activity against both soil-borne bacteria and fungi at the concentration detected in the root exudates.

KEYWORDS: ***Arabidopsis thaliana*; Brassicaceae; butanoic acid; *trans*-cinnamic acid; *o*-coumaric acid; *p*-coumaric acid; ferulic acid; *p*-hydroxybenzamide; methyl *p*-hydroxybenzoate; 3-indolepropanoic acid; syringic acid; vanillic acid; elicitation**

INTRODUCTION

The hidden half of a plant system thrives in a diverse, ever-changing environment with bacteria, fungi, and other microorganisms feeding on an array of organic material (*1*). Thus, the area of soil surrounding a plant root represents a unique physical, biochemical, and ecological interface between the roots and the external environment. This so-called rhizosphere is in part regulated by the root system itself through chemicals exuded/secreted into the surrounding soil. Root exudates include low molecular weight secondary metabolites and high molecular weight compounds such as mucilage and proteins (*2*). It is estimated that nearly 5−21% of photosynthetically fixed carbon is eventually transferred to the rhizosphere in the form of root exudates (*3*, *4*), but the mechanisms that drive and regulate this process are not fully understood.

Secondary plant metabolites may not play a fundamental biochemical role in the normal building and maintaining of plant cells (*5*), but they can clearly play an ecological role (*6*). Secondary metabolism is important in many plant processes, including root-specific metabolite production and root secretion (*7*, *8*). Elicitors are molecules that stimulate defense or stress-induced responses in plants (*9*). The detection of novel secondary metabolites upon root elicitation suggests that elicitation and secondary metabolite profiling may be harnessed as a functional tool to reveal silent genes in plant genomes (*10*, *11*).

Functional genomic approaches have been used to characterize the biological diversity and development of plants at the gene level. Proteomic and mRNA profilings complement functional genomics and provide information about the relationship between gene activation, transcription, and successful translation. Metabolic profiling aims to improve our understanding of the connection between genes, proteins, and plant metabolism (*12*−*15*). Accordingly, metabolic profiling will be extremely useful for studying changes in gene expression and the ensuing metabolic and phenotypic response during plant development or in response to environmental and/or chemical stress (*16*). Extensive primary metabolic profiling using gas chromatography and mass spectrometry of potato tubers from various transgenic potato lines (*13*) and *Arabidopsis thaliana* leaves (*12*) has allowed for the detection and quantification of more than 100 polar compounds (sugars and amino acids) within potato tubers and 300 polar compounds from *A. thaliana* leaf extracts. The use of data mining tools such as hierarchical cluster analysis and principle component analysis has revealed the presence of unique metabolic profiles for distinct genotypes, demonstrating the value of metabolic profiling as an additional tool in the characterization of plant genotypes (*12*, *13*).

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10.1021/jf021166h CCC: $25.00 © 2003 American Chemical Society
Published on Web 03/20/2003

Table 1. Characterization of 10 Secondary Metabolites Detected in the Root Exudates of A. *thaliana* As Determined by HPLC and NMR

Compound	Elicitor Treatment (Day); RT (mins)	Structure	λ-max (190-800 nm)	Reported Biological Activity	Reference
1	Salicylic acid (35); (57.483)	H_3C COOH	275	Allelopathic	(*33*)
2	Chitosan (7); (58.983)	COOH	245	Allelopathic	(*33*)
3	Jasmonic acid (21); (67.450)	COOH, OH	210	Antifungal	(*37*)
4	Jasmonic acid (21); (54.700)	HO, COOH	210	Allelopathic	(*29, 30*)
5	Salicylic acid (35); (37.350)	H_3CO, HO, COOH	245	Allelochemical Antibacterial Chemopreventative	(*33, 35, 36*)
6	Chitosan (7); (67.783)	HO, O, NH_2	250	Unknown	-
7	Control (14); (57.600)	HO, O, OCH_3	275	Antibacterial	(*39*)
8	Salicylic acid (35); (67.550)	COOH, N, H	210	Antibacterial	(*40*)
9	Chitosan (7); (48.483)	H_3CO, COOH, HO, OCH_3	235	Allelochemical	(*29, 30*)
10	Chitosan (7); (57.617)	H_3CO, COOH, HO	275	Allelochemical Antifungal Chemopreventative	(*29, 30*) (*32*) (*36*)

To explore two neglected areas in plant biology, root secretion and secondary metabolite studies, we developed a system to metabolically profile secondary metabolites secreted from the roots of *A. thaliana* during its 60-day life cycle. This model plant system has the potential to accurately characterize biologically active secondary metabolites secreted by roots due to developmental cues or in response to pathogen attack or other stresses (*17*). We here report the metabolic profiling and partial characterization of water-saturated hexane soluble compounds secreted from the roots of *A. thaliana*.

MATERIALS AND METHODS

Plant Material. Seeds of wild-type *A. thaliana* ecotype Columbia (Col-O) were obtained from Lehle Seeds (Round Rock, TX).

Plant Initiation and Growth Conditions. Seeds of *A. thaliana* were surface sterilized using sodium hypochlorite (0.3% v/v) for 10−12 min and then washed four times in sterile double-distilled water. Seeds were placed on static Murashige and Skoog basal media (*18*) and allowed to germinate for 10 days until roots and shoots emerged. Ten-day-old seedlings with only roots submerged were transferred to 50 mL culture tubes with 10 mL of liquid MS basal media. Plant cultures were maintained on an orbital platform shaker set at 90 rpm (Lab-Line Instruments, Inc., Melrose Park, IL) with a photoperiod of 16 h and 8 h dark at 25 ± 2 °C. The light intensity in the growth chamber was 24 μmol m^{-2} s^{-1}. One plant per tube was tested for every elicitor treatment.

Root Elicitation and Medium Extraction. *A. thaliana* plants with well-differentiated roots were subjected to multiple abiotic chemical elicitors and biotic fungal cell wall elicitors by supplementing the 10 mL of liquid Murashige and Skoog medium. Initial pilot experiments were performed to determine the final elicitor concentration for the treatments. Elicitor concentrations were selected on the basis of inducing maximum root secretion and avoidance of tissue toxicity. Roots were treated with the following: 100 μM salicylic acid, 800 μM jasmonic acid, 0.12% (w/v) chitosan, and fungal cell wall elicitors (CWE) from

Phytophthora cinnamoni (3 mL v/v), and *Rhizoctonia solani* (3 mL v/v). Solutions of salicylic acid and jasmonic acid were prepared in ethanol, chitosan was dissolved in 0.1 N acetic acid and then neutralized with 0.1 N sodium hydroxide to pH 5.8, and fungal cell wall elicitors were prepared and administered as previously described by McKinley et al. (*19*). A time course experiment was established, wherein medium samples from all of the treatments were extracted on days 7, 14, 21, 35, and 48 postelicitation. A nonelicited control was also analyzed during the same time period. Other control experiments included plain Murashige and Skoog basal liquid medium, elicitor standards, and ethanol treatments in the absence/presence of plants. Compounds detected in the different elicitation treatments with *A. thaliana* were not detected in any of the control experiments without plants. Exudates were checked for possible cross-contamination by plating on Luria Bertini (LB) and potato dextrose agar (PDA) media. The medium samples (3 mL) from all treatments were filtered through a nylon syringe filter of pore size 0.45 μm (Scientific Resources Inc., Duluth, GA) to remove any cellular debris and partitioned against 5 mL of hexane (Fisher Scientific, Pittsburgh, PA) for 24 h at room temperature. The hexane phase was concentrated by freeze-drying to remove volatiles and water (model 25LL, Vir Tis, Genesis), and the concentrate was dissolved in 200 μL of absolute methanol (Fisher Scientific).

High-Performance Liquid Chromatography (HPLC) Analysis of Root Exudates. Extracts from the media were injected into an HPLC system. Compounds in the root exudates were chromatographed by gradient elution on a 25 cm × 4.6 mm reverse phase, C_{18} column (Supelco Co., Bellefonte, PA). The chromatographic system (Dionex Co., Sunnyvale, CA) consisted of P580 pumps connected to an ASI-100 automated sample injector. Detection was at 210 nm with a PDA-100 photodiode array variable UV−vis detector (Dionex Co.). The injected samples (15 μL) were subjected to a broad-range wavelength scan between 190 and 800 nm. Mobile phase A consisted of double-distilled water and mobile phase B of acetonitrile (Fisher Scientific). A multistep gradient was applied for all separations with a flow rate of 1 mL min^{-1}. The multistep gradient was as follows: 0−5 min, 5.0% B; 5−10 min, 20.0% B; 15−20 min, 40.0% B; 20−40 min, 80.0% B; 40−60 min, 100% B; 60−70 min, 100% B; 70−80 min, 5.0% B.

^{1}H and ^{13}C NMR. Peaks collected from HPLC runs were used for ^{1}H and ^{13}C NMR analysis. ^{1}H and ^{13}C NMR analyses were conducted at ambient temperature using an INOVA 300 MHz Fourier transform spectrometer (Varian Inc., Harbor City, CA). The spectra were identified by comparison with published spectra in *The Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra* (*20*) and also compared to commercial samples (Sigma Chemical Co., St. Louis, MO).

Bioassay Procedures. Both fungal and bacterial isolates from a broad phylogenetic range were tested for inhibition of growth with authentic compounds **1**−**10** (Sigma Chemical Co.). All compounds were tested at the concentration detected in the root exudates and/or at half the detected concentration. Initial stock solutions (1 mg mL^{-1}) of each compound were prepared in methanol. Inhibition of hyphal growth in *Fusarium oxysporum*, *Phytophthora drechsleri*, and *Rhizoctonia solani* was tested by linear growth assay. Fungal isolates were maintained on PDA in the dark at 24 °C. Compounds were applied to sterile filter disks and allowed to air-dry before being arranged in a circle on a 35 mm Petri dish. A 4 mm plug of fungal hyphae was placed in the center of the Petri dish, and inhibition was observed on a daily basis. Each fungal isolate was tested against all compounds and concentrations in two separate replicates. Bacterial assays were performed in 96-well, sterile, flat-bottom microtiter plates (Nalge Nunc International, Roskilde, Denmark). Bacterial suspension cultures of *Erwinia carotovora*, *Erwinia amylovora*, *Xanthomonas campestris* pv *vesicatoria*, and *Pseudomonas fluorescens* were grown overnight at 37 °C to OD_{600} = 0.2. Test wells contained 100 μL of the tested bacteria in combination with the standard compound at the concentration tested. Control wells contained 100 μL of bacteria alone with the highest volume of methanol used. The plates were covered with sterile lids and placed in polystyrene boxes lined with moistened filter paper to maintain high humidity and incubated at 37 °C. The absorbance of each well was determined at OD_{600} with an Opsys MR, microtiter plate reader (Dynex Technologies, Chantilly, VA) at the beginning of the assay and after 24 h of incubation. Each bacterial isolate was tested against all compounds and concentrations in two separate replicates. Net bacterial growth was calculated by subtracting the initial OD_{600} from the OD_{600} after 24 h of incubation. Percent inhibition (%I) was calculated using net bacterial growth based on OD_{600} readings with the following formula: (untreated − treated/untreated) × 100.

Table 2. Antifungal Activities of the 10 Characterized Secondary Metabolites[a]

treatment (μM)	*F. oxysporum*	*P. drechsleri*	*R. solani*
control[b]	–	–	–
butanoic acid (52.60)	–	–	–
butanoic acid (105.35)[c]	–	–	–
trans-cinnamic acid (0.67)	–	–	+
trans-cinnamic acid (8.09)[c]	–	–	+++
o-coumaric acid (2.05)	–	–	+
o-coumaric acid (4.10)	–	–	+
o-coumaric acid (6.80)[c]	–	–	++
p-coumaric acid (0.60)	–	–	–
p-coumaric acid (1.20)[c]	–	–	–
ferulic acid (38.60)	+++	–	–
ferulic acid (76.70)[c]	+++	–	–
p-hydroxybenzamide (3.85)	+	+	+
p-hydroxybenzamide (13.61)	++	++	++
p-hydroxybenzamide (16.30)[c]	++++	+++	++++
methyl *p*-hydroxybenzoate (26.21)	–	–	–
methyl *p*-hydroxybenzoate (125.51)[c]	–	–	–
3-indolepropanoic acid (30.61)	–	–	–
3-indolepropanoic acid (61.80)[c]	–	–	–
syringic acid (0.75)	–	–	–
syringic acid (1.46)[c]	–	–	–
vanillic acid (9.20)	++	++	+
vanillic acid (25.60)	++	++	++
vanillic acid (42.06)[c]	++++	++++	++++

[a] Data are presented as follows: –, no inhibition of fungal growth; +, slight inhibition; ++, weak inhibition; +++, moderate inhibition; ++++, strong inhibition; all points are the average of two separate experiments with two replicates in each treatment. In the table, each + represents 5 mm from the filter disk; – depicts no fungal inhibition. All antifungal experiments were performed with standard, commercially available compounds. [b] Control disks contained the highest volume of methanol used for each treatment. [c] Biological concentrations of the compound found in the exudates.

RESULTS

In Vitro Experimental System. An in vitro system implementing root-specific elicitation was developed to metabolically profile and characterize root-secreted secondary metabolites from *A. thaliana*. Nonelicited control plants were maintained under the same conditions as elicited plants, but no elicitor was exogenously added to the liquid medium of the control plants. Additionally, medium exudates from all treatments on corresponding harvest days were plated on both LB and PDA media to check for possible microbial contamination that would produce metabolites unrelated to the metabolites secreted by the plant roots; however, we did not observe any microbial contamination in any of the treatments.

Quantification of Total Secondary Metabolites Secreted by *A. thaliana* Roots. To initially quantify the total number of rhizosecreted secondary metabolites from the roots of *A.*

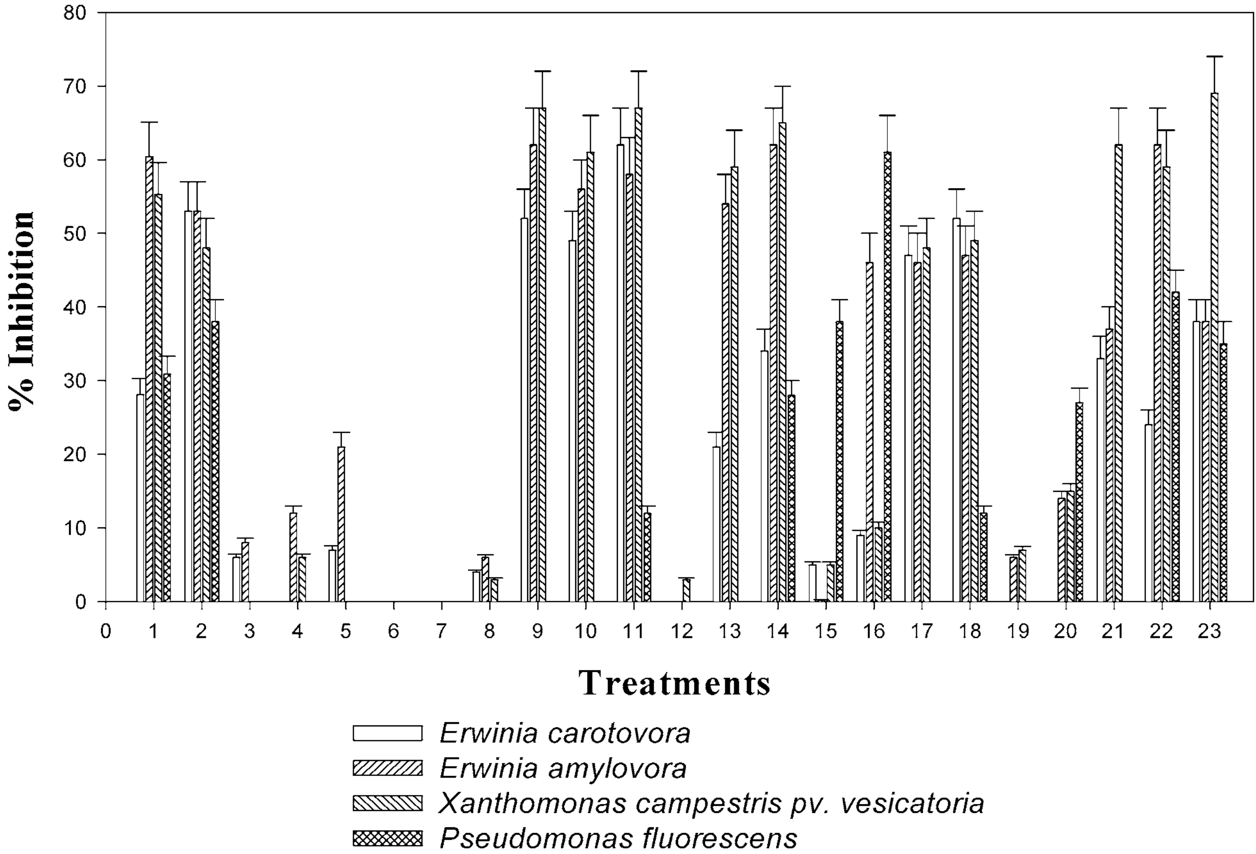

Figure 1. Antibacterial activities of the 10 characterized secondary metabolites on different bacterial strains: (1) butanoic acid (52.60 μM); (2) butanoic acid (105.35 μM); (3) *o*-coumaric acid (2.05 μM); (4) *o*-coumaric acid (4.10 μM); (5) *o*-coumaric acid (6.80 μM); (6) *p*-coumaric acid (0.60 μM); (7) *p*-coumaric acid (1.20 μM); (8) *trans*-cinnamic acid (0.67 μM); (9) *trans*-cinnamic acid (8.09 μM); (10) ferulic acid (38.60 μM); (11) ferulic acid (76.70 μM); (12) *p*-hydroxybenzamide (3.85 μM); (13) *p*-hydroxybenzamide (13.61 μM); (14) *p*-hydroxybenzamide (16.30 μM); (15) methyl *p*-hydroxybenzoate (26.21 μM); (16) methyl *p*-hydroxybenzoate (125.51 μM); (17) 3-indolepropanoic acid (30.61 μM); (18) 3-indolepropanoic acid (61.80 μM); (19) syringic acid (0.75 μM); (20) syringic acid (1.46 μM); (21) vanillic acid (9.20 μM); (22) vanillic acid (25.60 μM); (23) vanillic acid (42.06 μM). Net bacterial growth was calculated by subtracting the OD_{600} at the beginning from the OD_{600} after 24 h of incubation. Percent inhibition (%I) was calculated using net bacterial growth based on OD_{600} readings with the following formula: (untreated − treated/untreated) × 100. Values are mean ± SD (n = 5). The highest concentration referred to in all treatments represents the approximate biological concentration in the root exudates of *A. thaliana*.

thaliana, medium samples from six treatments [salicylic acid, jasmonic acid, chitosan, and fungal cell wall elicitors (CWE) from *Phytophthora cinnamoni* and *Rhizoctonia solani*] and a nonelicited control were analyzed by HPLC on days 7, 14, 21, 35, and 48 postelicitation. Individual treatments had six replicates, and the experiment was repeated twice to confirm the reproducibility of the results. After HPLC analysis, a total of 289 secondary metabolite compounds were quantified using the spectrophotometric response. The first compound eluted at time 1.11 min of the 80 min HPLC gradient, and the last compound eluted at time 74.85 min. The nonelicited control secreted 68 of the 289 compounds over the time course study, which was the maximum number of compounds detected in any of the six treatments. Among the five elicitor treatments, roots elicited with 100 μM salicylic acid and 3 mL of *R. solani* (v/v) secreted the maximum number of compounds (66), whereas roots elicited with 800 μM jasmonic acid secreted the minimum number of compounds (52) over the time course study. The HPLC profile of root tissues was different in quality and quantity from the profile of the root exudates, indicating that root secretion of secondary metabolites is possibly an active process dependent on various stress factors (data not shown).

In addition to production variations within the six treatments over the time course study, the number of compounds secreted on individual harvest days (7, 14, 21, 35, and 48 days postelicitation) within the six treatments also varied. In the nonelicited control, a maximum number of compounds (28) was detected on day 7, whereas a minimum number (6) was detected on day 48 when plants were beginning to senesce. Additionally, it was observed that elicitation influenced the number of compounds secreted on each harvest day compared to the control treatment. The maximum number of compounds (32) was detected on day 35 of the salicylic acid treatment, whereas a minimum number (4) was detected on day 35 of the jasmonic acid treatment. We witnessed a similar pattern of secretion in all of the elicitor treatments, wherein a maximum number of compounds was detected on either day 21 or day 35 and fewer compounds were detected on days 7, 14, and 48. In contrast, the nonelicited control treatment secreted a maximum number of compounds on day 7 and a lesser number of compounds on day 21 (14) and day 35 (13), respectively. The presence of an increased number of compounds in the root exudates on day 21 or 35 suggests elicitation with chemical and fungal cell wall elicitors results in a coordinated defense response by the root system.

Time Course Persistence and Rhizosecretion of Secondary Metabolites. Using our in vitro system and HPLC analysis, we monitored on a nearly weekly basis the persistence of secreted compounds in the media and quantified newly secreted compounds. The majority of compounds did not persist continually

in the root exudates and were not detected at each harvest day. Additionally, all elicitor standards were subjected to HPLC analysis to determine retention times, but these retention times were not detected in any of the profiles from the treatments.

Characterization of 10 Secondary Metabolites. After visual examination of the complete set of HPLC chromatograms, it was apparent that the roots of *A. thaliana* were secreting several common and distinctive secondary metabolites (data not shown). Peaks were selected according to several criteria: the compounds displayed characteristic production within an elicitor treatment; the compounds appeared on a specific harvest day(s), and the concentration of the compound was enhanced compared to the other compounds secreted. We characterized 10 compounds on the basis that they are potentially elicitor-induced stress/defense compounds that may possess the greatest biological activity when secreted into the rhizosphere. From the HPLC elutants, the 10 compounds were collected and analyzed by ^{1}H and ^{13}C NMR spectroscopy. The following compounds were identified: butanoic acid, **1**; *trans*-cinnamic acid, **2**; *o*-coumaric acid, **3**; *p*-coumaric acid, **4**; ferulic acid, **5**; *p*-hydroxybenzamide, **6**; methyl *p*-hydroxybenzoate, **7**; 3-indolepropanoic acid, **8**; syringic acid, **9**; and vanillic acid, **10**. All of the compounds with the exception of **7** were isolated from elicitor treatments. **Table 1** summarizes the 10 characterized metabolites including chemical structure, elicitor treatment and harvest day, HPLC retention time, λ_{max}, and reported biological activity.

Antifungal and Antibacterial Activities of 10 Characterized Secondary Metabolites. We tested the 10 secondary metabolites for antimicrobial activity against an array of root-colonizing fungal and bacterial isolates using commercially available standards of the 10 compounds at the concentration detected in the root exudates and/or half the detected concentration. The antifungal activities of the 10 compounds against *Fusarium oxysporum*, *Phytophthora drechsleri*, and *Rhizoctonia solani* are shown in **Table 2**. Of the compounds tested, only *p*-hydroxybenzamide (3.85, 13.61, and 16.30 μM) and vanillic acid (9.2, 25.6, and 42.06 μM) were active against all three fungi (**Table 2**).

The antibacterial activities of the 10 compounds were investigated in vitro against *Erwinia carotovora*, *Erwinia amylovora*, *Xanthomonas campestris* pv *vesicatoria*, and *Pseudomonas fluorescens*. The 10 compounds displayed various antibacterial activities (**Figure 1**). With the exception of **7** (30.61 and 61.80 μM) and **9** (1.49 μM) treatments, *P. fluorescens* was more resistant to exuded compounds than the other three bacteria (**Figure 1**).

DISCUSSION

Our studies revealed that secondary metabolites previously undocumented in the root exudates of *A. thaliana* can be identified and characterized using HPLC and ^{1}H and ^{13}C NMR. Metabolic profiling with mass spectrometry has been used primarily in medical analysis and in the detection of human diseases (*21*−*23*), with only a few studies using these techniques to investigate plant systems (*12*, *13*). We studied the model plant system *Arabidopsis* because it has been well characterized at the genomic, biochemical, and metabolic levels. Moreover, we have observed that root secondary metabolite secretion in vitro is comparable with the secretion in the soil in different plant systems (*7*, *24*), thus validating the broader application of our experimental approach.

Although the major purpose of this investigation was to study the effect of different elicitors on secondary metabolite secretion in the root exudates of *A. thaliana*, the preliminary analysis allowed for some important conclusions. For example, the detection of 289 possible secondary metabolites in the root exudates of *A. thaliana* and only 68 compounds in the nonelicited control suggests that root exudation is a selective process. Additionally, the quantitative and qualitative variation in the concentration of certain metabolites within the six treatments at different time points during the time course reconfirms the concept that roots respond differently under different stress conditions. Some plausible mechanisms that may account for the observed variations include the age of the plant in conjunction with the type of elicitor treatment, the instability of the secreted metabolites due in part to root enzymatic activity aimed at avoiding possible toxicity, or the recycling of rhizosecreted secondary metabolites by the root system.

Unlike the secretion of secondary metabolites in trichomes from plant leaves (*25*), the secretion of secondary metabolites from roots and the cellular mechanisms that drive root secretion are poorly understood. However, previous studies on various plant species have described the increased production and exudation of specific root secondary metabolites (*7*, *10*, *11*, *24*, *26*), but to our knowledge no studies have profiled and characterized root exudates in *A. thaliana*.

Although most of the 10 compounds identified are known chemically (**Table 1**), the metabolites have not been previously detected in root exudates of *A. thaliana*. In our studies, the 10 identified metabolites were secreted under different elicitor conditions (**Table 1**), suggesting that the root system is capable of adapting as the rhizospheric conditions change. This finding also demonstrates the potential usefulness of secondary metabolic profiling in functional genomics, because it could lead to the utilization of a direct functional link between gene activation in the phenylpropanoid pathway, production of bioactive metabolites, and disease/insect resistance in this species (*27*). To our knowledge, only the metabolites **2**, **4**, and **5** have been reported in *A. thaliana*, whereas **1**, **3**, and **6**−**10** have never before been reported from this species. *p*-Coumaric acid (**4**) has been reported in *A. thaliana* (*28*) and is an active allelochemical isolated from both shoot and root tissues in wheat (*Triticum aestivum* L.) (*29*, *30*). El Modafar and El Boustani (*31*) detected **4** in high concentrations in the root tissue of cultivars of date palms resistant to *F. oxysporum*. Both *Fusarium* spore germination and mycelial growth were significantly inhibited by **4** (*32*), but we did not observe any activity with **4** at the biological concentrations detected in *A. thaliana*'s root exudates (**Table 1** and **Figure 1**). Because **4** was isolated from the 800 μM jasmonic acid elicitor treatment on day 21 (**Table 1**) and was not detected on any other harvest day or in any of the other treatments, we suggest that **4** is a jasmonic acid-induced metabolite that is not constitutively secreted and may have specific bioactivity. Although there are no known reports of any biological activity of *trans*-cinnamic acid (**2**) in *A. thaliana*, Chaves et al. (*33*) reported that this acid isolated from *Cistus ladanifer* leaf extracts had moderate allelopathic activity. In our study, **2** was isolated on day 7 from the 0.12% w/v chitosan treatment (**Table 1**) and was also detected at considerably lower concentrations on day 14 of the chitosan treatment and on day 35 of the *P. cinnamoni* treatment. *trans*-Cinnamic acid had moderate antifungal activity against *R. solani* (**Table 1**), and it was inhibitory against all bacteria tested except *P. fluorescens* (**Figure 1**). The presence of ferulic acid (**5**) and its role in lignin biosynthesis have been well documented in *A. thaliana* (*34*). Some reported biological activities of isolated ferulic acid in other plants include allelochemical (*33*), antibacterial (*35*), and

chemopreventative (*36*). In our studies, **5** displayed moderate antifungal activity against *F. oxysporum* only (**Table 2**) and antibacterial activity against all bacteria tested. A high concentration of **5** on day 35 of the 100 μM salicylic acid treatment only (**Table 1**) suggests that it is an salicylic acid-induced secondary metabolite.

Butanoic acid (**1**) from leaf extracts of *Cistus ladanifer* displayed high allelopathic activity against *Rumex crispus* seedlings (*33*). We detected a high concentration of **1** on day 35 in the salicylic acid treatment (**Table 1**) and showed that at the concentration detected in the root exudates it has strong antibacterial activity (**Figure 1**). *p*-Coumaric acid (**4**) has been well documented in *A. thaliana* (*28*) but not *o*-coumaric acid (**3**). We isolated **3** from the jasmonic acid treatment on day 21 and in the *R. solani* treatment on day 21 (**Table 1**). *o*-Coumaric acid (**3**) isolated from olive callus tissue has shown antifungal activity against *Aspergillus flavus* by inhibiting aflatoxin production (*37*). Even at very low concentrations, **3** has antifungal activity against *R. solani* (**Table 2**) and slight antibacterial activity (**Figure 1**). It is interesting to note the differences in bioactivities between the two regioisomers, **3** and **4**. Syringic acid (**9**) has been isolated from both the shoot and root tissues of wheat and has been identified as a potent allelochemical (*29*, *30*). We detected **9** at very low concentrations on day 7 of the chitosan treatment (**Table 1**) and saw slight antibacterial activity with **9** (**Figure 1**). Although we are reporting vanillic acid (**10**) for the first time in the root exudates of *A. thaliana*, it is a well-documented phenolic acid in other plant species with an assortment of biological activities (**Table 1**). We detected **10** on days 7 and 14 in the chitosan treatment and also detected higher concentrations on day 7 in the *R. solani* treatment (**Table 1**). Vanillic acid was inhibitory against all three fungal pathogens (**Table 2**) and showed activity at the higher concentrations tested against all bacteria (**Figure 1**), which suggests that its secretion is triggered by the presence of fungal or even bacterial pathogens.

p-Hydroxybenzamide (**6**) was isolated on day 7 from the chitosan treatment, and we additionally detected it on day 14 of the chitosan and jasmonic acid treatments (**Table 1**). The occurrence of **6** in the exudates of the chitosan and jasmonic acid treatments and the antifungal and antibacterial activities of this compound (**Table 1** and **Figure 1**) suggest that this previously unreported plant compound might be important in plant defense. Methyl *p*-hydroxybenzoate (**7**), apparently not reported before in plants, is a common component (as methyl paraben) of insect artificial diets (*38*). It was also active against the bacteria we tested (**Figure 1**), supporting its use as a common food preservative (*39*). Methyl *p*-hydroxybenzoate was isolated from the nonelicited control treatment on day 14, which indicates that it is a constitutively secreted compound, but it was also detected at higher concentrations in the *P. cinnamoni* treatment (**Table 1**). *A. thaliana* and other members of the Brassicaceae family are known for the occurrence of various indolic constituents including several indolyl glucosinolates (*27*), but no references to 3-indolepropanoic acid (**8**) as a plant isolate were found. Matsuda et al. (*40*) showed that synthetic **8** was active against *Pseudomonas solanacearum*, which accords with our reported antibacterial activity including *P. fluorescens* (**Figure 1**). Because many of the compounds tested displayed both antifungal and antibacterial activity, we believe that several secreted compounds, rather than a single one, account for the overall antimicrobial activity of *Arabidopsis*.

It is generally believed that the total number of compounds present in all of the plant kingdom approaches 200,000, and nearly 5000 compounds may potentially be found in *Arabidopsis*, with secondary metabolism having a significant role in this chemical diversity (*5*). Therefore, our approach does not signal the end to further profiling of root exudates of *A. thaliana*. We feel metabolic profiling and characterization of root exudates will aid in understanding the complex interactions within the rhizosphere. The occurrence of enhanced exudation of specific compounds upon various elicitor treatments suggests a possible interaction between plant roots and microbes. Finally, because the functions of nearly 35% of the genes in *Arabidopsis* are unclassified (*41*), profiling of secondary metabolites has the potential to be used in reverse metabolomics to isolate novel genes involved in secondary metabolism.

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Received for review November 27, 2002. Revised manuscript received February 6, 2003. Accepted February 7, 2003. Work reported in this paper was supported by the Colorado State University Agricultural Experiment Station (J.M.V. and F.R.S.) and the Colorado State University-Invasive Weeds Initiative (J.M.V. and F.R.S.). J.M.V. is an NSF Early Career Development Faculty Fellow.

JF021166H