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Proteome of Soybean Seed Exudates Contains Plant Defense-Related Proteins Active against the Root-Knot Nematode *Meloidogyne incognita*

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ABSTRACT: Several studies have described the effects of seed exudates against microorganisms, but only few of them have investigated the proteins that have defensive activity particularly against nematode parasites. This study focused on the proteins released in the exudates of soybean seeds and evaluated their nematicidal properties against *Meloidogyne incognita*. A proteomic approach indicated the existence of 63 exuded proteins, including β-1,3-glucanase, chitinase, lectin, trypsin inhibitor, and lipoxygenase, all of which are related to plant defense. The presence of some of these proteins was confirmed by their *in vitro* activity. The soybean exudates were able to reduce the hatching of nematode eggs and to cause 100% mortality of second-stage juveniles (J2). The pretreatment of J2 with these exudates resulted in a 90% reduction of the gall number in tobacco plants. These findings suggest that the exuded proteins are directly involved in plant defense against soil pathogens, including nematodes, during seed germination.

KEYWORDS: *Glycine max, exudation, seed proteins, defense proteins, soil pathogen, Meloidogyne*

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are obligate endoparasites comprising a major group of plant-parasitic nematodes that affect crop production, causing annual losses of more than one hundred billion U.S. dollars worldwide.[1] Among all *Meloidogyne* species, *Meloidogyne incognita* is the most damaging phytonematode, and it is disseminated throughout the world, particularly in regions with previous plantings of coffee or cotton.[2] Root-knot nematodes have developed a highly specialized and unique way to infect their hosts. The infection takes place when the motile second-stage juvenile (J2), the infective form, is attracted to the root system by the root exudates and enters the elongation zone just behind the root tip.[3] The parasite then moves intra- and intercellularly to reach the primary phloem or the undifferentiated cells of the adjacent parenchyma, where it becomes sedentary and establishes a feeding site.[4]

Currently, the most commonly used procedure in nematode control is the application of synthetic nematicides. However, their high cost and toxicity to the environment and live organisms has encouraged the development of new methods of nematode control.[5] Although transgenic technology, which has offered potential new avenues for crop improvement programs, is useful for designing nematode-resistant crops, this alternative approach has not sufficiently demanded an urgent need for the design of novel management strategies.[6] In this sense, a number of studies have been performed to develop non-chemical and environmentally friendly nematode management practices, including the use of botanicals.[7]

Seed exudates represent a useful source of defense molecules, which are mainly peptides/proteins and secondary metabolites with the capacity to inhibit soil-borne pathogens and, thereby, prevent infection.[8] Although several studies have described the inhibitory effects of seed exudates against microorganisms, only a few of these studies have investigated the proteins that may contribute to the defense activity of the exudates; thus, our knowledge remains essentially incomplete.[9] The present study focused exclusively on the exuded proteins from soybeans [*Glycine max* (L.) Merril]. Soybean seeds stand as an ideal system for the study of the exudates−pathogen interaction because of their great economic value and high protein content.[10] Many seed proteins are usually stored in specialized cellular compartments, preventing their hydrolysis.[11] In addition, it was shown that soybean seeds are capable of exuding large amounts of protease inhibitors along with other proteins in hot water, suggesting their potential role in the defense against pathogens.[12] In this work, the identification and

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characterization of some proteins were performed by studying the soybean seed exudates using capillary liquid chromatography/nanoelectrospray ionization tandem mass spectrometry (ESI−LC−MS/MS) in association with data searching for protein identification and by measuring the activity of some classical defense proteins. Moreover, the efficiency of the exuded seed proteins in inhibiting the development of *M. incognita* was investigated. This study was performed to contribute to the understanding of the physiological role of exuded proteins in the defense against soil pathogens, particularly during the germination process as well as to demonstrate the potential of seed exudates as a source of nematicidal compounds.

MATERIALS AND METHODS

Biological Materials and Reagents. Mature soybean seeds cv. BRS Pala, a commercial and conventional soybean cultivar with high protein content (38.8%) and good resistance/tolerance to both biotic and abiotic stresses, were supplied by the Brazilian Agricultural Research Corporation [Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)] in partnership with the Foundation for ProSeed Research Support, Passo Fundo, Brazil.[13] Rabbit blood was obtained from the animals of colonies maintained at the Federal University of Ceará (Fortaleza, Brazil). *M. incognita* (race 1) eggs were isolated from tomato roots growing in a greenhouse at the Plant−Pest Molecular Interaction Laboratory, Embrapa, Brasília, Brazil. All chemicals used were of analytical grade.

Preparation of the Soybean Seed Exudates. Seed exudates were obtained from 20 whole soybean seeds with no cracks or other injuries. Seeds were surface-sterilized with 30% (v/v) ethanol for 5 min, rinsed exhaustively with distilled water, and soaked in 6 mL of 0.1 M sodium acetate buffer at pH 5.0, 28 °C, and 70% relative humidity. After different time periods of soaking (1, 6, 12, 18, and 24 h), the seeds were removed, the exudates were collected, and the volumes were recorded. The soybean seed exudates were subject to exhaustive dialysis against distilled water to remove organic compounds with a molecular mass below 3500 Da and centrifuged at 8000*g* for 10 min at 4 °C; the supernatant was collected; and its protein content was determined by the Bradford[14] method using bovine serum albumin (BSA) as the standard protein.

Phytochemical Compound Analysis. The presence of phytochemical compounds was assessed[15] in the ethanolic (70%) extract obtained from the lyophilized soybean exudates. To detect phenols and tannins, the extract was mixed with a few drops of alcoholic ferric chloride solution. Color varying between blue and red shows the presence of phenols, and a dark blue or green precipitate denotes tannins. Leucoanthocyanidins were detected by the appearance of red color after acidification of the extract with HCl (pH 1−3), followed by heat treatment for 2−3 min. To identify flavonoids and xanthones, the extract was mixed with magnesium granules in concentrated HCl. The appearance of red color indicates the presence of these compounds. In the test for steroids and triterpenoids, the extract was mixed with chloroform, a few drops of acetic anhydride, and concentrated sulfuric acid. The appearance of blue or green color indicates the presence of steroids, and brown to red color indicates the occurrence of triterpenoids. The test for saponins was performed in the precipitate formed after treatment of the extract with chloroform, followed by the addition of distilled water and vigorous shaking. The appearance of stable froth indicates the presence of saponins. Alkaloids were detected after extraction of the organic bases with diluted HCl, followed by the addition of drops of the Hagger, Mayer, and Dragendorff reagents. The appearance of cream white precipitate indicates the presence of alkaloids.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) Analysis. SDS−PAGE was performed in a 12.5% discontinuous polyacrylamide gel prepared in 0.025 M Tris−HCl buffer at pH 8.9 containing 1% sodium dodecyl sulfate (SDS).[16] Samples (25 μg) were prepared in 0.5 M Tris-HCl buffer at pH 6.8 containing 1% SDS and heated at 100 °C for 10 min before electrophoresis. Electrophoresis was performed at 20 mA, and the protein bands were visualized after staining with Coomassie Brilliant Blue G-250.[17]

ESI−LC−MS/MS Analysis and Database Searching. The 50 μg aliquots of the dialyzed soybean seed exudates were dissolved in 0.05 M ammonium bicarbonate, denatured in the presence of 0.2% (v/v) RapiGEST SF (Waters, Manchester, U.K.) at 80 °C for 15 min in a dry bath, reduced with 0.1 M dithiothreitol (DTT) at 60 °C for 60 min, and alkylated with 0.3 M iodoacetamide (IAA) for 30 min in the dark at room temperature (23 ± 2 °C). Next, the samples were enzymatically digested overnight, at 37 °C, with the addition of a modified trypsin (Promega, catalog number V5111, Madison, WI) at a 1:100 (w/w) enzyme/protein ratio. To hydrolyze RapiGEST SF and stop digestion, 10 μL of 5% trifluoroacetic acid (TFA) was added, the sample was mixed, incubated for 90 min at 37 °C, and centrifuged (14000*g* for 30 min at 6 °C), and the supernatant was transferred to a Waters Total Recovery vial (Waters, Manchester, U.K.). The nano-ultra-performance liquid chromatography (UPLC) separation of tryptic peptides was performed using a nanoACQUITY UPLC system (Waters, Manchester, U.K.) equipped with a HSS T3 C_{18} reverse-phase column (1.8 μm, 75 μm × 20 mm) equilibrated with the mobile phase A [0.1% (v/v) formic acid in water]. Elution was carried out at 0.35 μL/min flow rate using the mobile phase B [0.1% (v/v) formic acid in acetonitrile] in a gradient mode of 0−40% for 90 min and 40−85% for 5 min. All samples were measured using a data-independent analysis (MS^E, where E represents dynamically variable collision energy), and the tryptic peptides were analyzed using a nano-ESI−LC−MS/MS mass spectrometer (Synapt G1 HDMS, Waters, Manchester, U.K.). The instrument was operated in the electrospray positive-ion mode nano-ESI (+) and in "V" mode, with a precursor double-charge resolution of at least 10 000 full width at half-maximum (fwhm). The mass spectrometer was calibrated by infusion of the standard [Glu1]−fibrinopeptide B human (Glu−Fib) ($[M + 2H]^{2+}$ = 785.2486) into the NanoLockSpray source. Analyses were performed using a Glu−Fib mass channel for every 30 s. The exact mass retention times (EMRTs) of nano-LC−MS^E data were collected in scans alternating between lower (3 eV) and elevated (15−50 eV) collision ramp energy applied to the argon collision cell. The scan time was 1.5 s with a 0.2 s interscan delay for each MS scan at m/z 50−2000. The radio frequency (RF) offset (MS profiles) was adjusted such that LC−MS data were effectively acquired from m/z 300 to 2000, which ensured that any masses observed in the LC−MSE data less than m/z 300 were known to arise from dissociations in the collision cell. The LC−MSE data were processed, and the proteins were identified using Protein Lynx Global Server, version 2.4, software (PLGS) against a reverse *G. max* annotated database, which is accessible on the Universal Protein Knowledgebase (UniProtKB) database. For spectra and database searching, the following default parameters on PLGS were used: a maximum of 1 missed cleavage by trypsin, a fixed modification specified as carbamidomethylation (C), and oxidation (M) as variable modification. The identified proteins were analyzed using AgBase tools.[18] The Plant GOSlimViewer was used to summarize the molecular function and biological process categories using UniProtKB as the reference. The identified proteins were further analyzed by TargetP,[19] iPSORT,[20] and SignalP 3.0,[21] three independent software programs that predict the secreted proteins into the medium via a classical pathway based on their N-terminal signal peptide sequences. In addition, the predictive characterization of proteins secreted via non-conventional pathways was performed using the neural network-based software SecretomeP, version 2.0.[22]

Activity Assay of Plant Defense-Related Proteins. β-1,3-Glucanase (EC 3.2.1.39) was measured by determining the amount of glucose liberated by the action of the enzyme on laminarin (Sigma, St. Louis, MO) used as a substrate. After the addition of the appropriate reagents to the reaction mixture, according to Boller,[23] absorbance readings were taken at 520 nm and the amount of reducing sugars liberated was calculated on the basis of a standard curve created with known amounts (7.5−240.0 mg/L) of commercial glucose (Sigma Chemical Company) dissolved in 0.05 M sodium acetate buffer (pH

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5.2) containing 0.5 M NaCl. The activity was expressed as nanokatals per milligram of protein (nkat/mg of protein), with 1 nkat defined as 1.0 nmol of D-glucose released from laminarin per second at 37 °C. Chitinase (EC 3.2.1.14) was assayed using a colorimetric procedure that detects *N*-acetyl-D-glucosamine (NAG) produced by the combined hydrolytic action of chitinases and β-glucuronidase upon the non-radioactive substrate colloidal chitin.[23,24] Colloidal chitin was prepared using non-radioactive acetic anhydride.[25] Absorbance was read at 585 nm, and the amount of NAG produced was calculated on the basis of a standard curve obtained with known concentrations (0.1−0.7 mg/L) of commercial NAG dissolved in 0.05 M sodium acetate buffer (pH 5.2). Chitinase was calculated as nanokatal per milligram of protein (nkat/mg of protein), with 1 nkat defined as 1.0 nmol of NAG produced per second at 37 °C. Peroxidase (EC 1.11.1.7) activity was determined using guaiacol as the proton donor substrate and hydrogen peroxide as the electron donor.[26] Aliquots of 0.02 mL of the dialyzed seed exudates appropriately diluted were mixed with 0.98 mL of 0.05 M sodium acetate buffer at pH 5.2, 0.5 mL of 0.06 M H_2O_2, and 0.5 mL of 0.02 M guaiacol in a total volume of 2.0 mL. The reaction mixture was incubated at 30 °C for 10 min, and the absorbance was measured at 480 nm. The variation of 1 unit of absorbance per minute was defined as 1 unit of peroxidase activity (1 AU) and expressed as activity per milligram of protein (AU/mg of protein). Total proteolytic activity was measured using azocasein as a non-specific substrate.[27] The soybean exudates (500 μL), at different concentrations, dissolved in 0.025 M Na−phosphate buffer at pH 6.0 containing 0.003 M DTT were incubated with 200 μL of 1% (m/v) azocasein for 1 h at 37 °C. The reaction was stopped by the addition of 300 μL of 20% (v/v) trichloroacetic acid (TCA). The tubes were centrifuged (5000*g* for 10 min at 25 °C), and the supernatants obtained were alkalinized with 400 μL of 0.2 N NaOH. The proteolytic activity was expressed as activity unit per milligram of protein per minute (AU mg^{-1} of protein min^{-1}), with 1 AU defined as the change of 0.01 absorbance unit per minute at 420 nm. The trypsin inhibitor assay was carried out by the method originally described by Kakade et al., using N_α-benzoyl-D,L-arginine 4-nitroanilide hydrochloride (L-BAPNA) as the substrate.[28,29] Seed exudates (0.1 mL) at different protein concentrations were mixed with 1.6 mL of 0.05 M Tris−HCl at pH 8.2 containing 0.02 M $CaCl_2$, with 0.1 mL of trypsin (Sigma, type I), from a stock solution of 0.4 mg in 10 mL of 0.001 M HCl, plus 0.1 mL of BAPNA [10 mg/mL in 1% (v/v) dimethyl sulfoxide (DMSO) plus H_2O]. The mixture was incubated for 45 min at 37 °C, and 0.2 mL of 30% (v/v) acetic acid solution was added. The enzymatic hydrolysis of BAPNA was evaluated by recording the absorbance at 410 nm. The amount of trypsin inhibited was calculated from a calibration curve using soybean trypsin inhibitor (Sigma, type I-S). The trypsin inhibitory activity was expressed as the amount of trypsin inhibited per milligram of protein (mg of TI/mg of protein). Papain inhibitory activity was performed as described by Abe et al.[30] using N_α-benzoyl-D,L-arginine-*p*-naphthylamide (BANA) as the substrate. To 20 μL of papain solution (0.1 g/L in 0.025 M sodium phosphate buffer at pH 6.0), 40 μL of an activation solution [containing 0.002 M ethylenediaminetetraacetic acid (EDTA) and 0.003 M DTT at pH 6.0], 100 μL of the dialyzed seed exudates at different concentrations, and 340 μL of 0.025 M sodium phosphate buffer at pH 6.0 were added. The mixture was incubated for 10 min at 37 °C. The reaction was initiated by the addition of 200 μL of 0.001 M BANA solution, prepared in 1% (v/v) DMSO and 0.025 M sodium phosphate buffer at pH 6.0. After 20 min at 37 °C, the reaction was stopped by the addition of 500 μL of 2% HCl in 95% ethanol. The color product was developed by adding 500 μL of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol. Papain inhibitory activity was expressed as inhibition unit per milligram of protein (IU/mg of protein), with 1 IU defined as the amount of inhibitor capable of decreasing the absorbance in 0.01 at 540 nm. Lectin activity was assessed by serial 2-fold dilution of samples, employing native and trypsin-treated rabbit red blood cells.[31,32] The seed exudates were diluted with 0.15 M NaCl in glass tubes and mixed with rabbit erythrocytes (2% suspension prepared in 0.15 M NaCl). The red cell suspensions were enzyme-treated with trypsin (10 mg/L solution prepared in 0.15 M NaCl) for 1 h at room temperature (22 ± 3 °C). Cells were washed 4 times with 0.15 M NaCl, and the suspension final volume was adjusted to attain the original concentration (2%). The degree of agglutination was monitored visually after the tubes had been left to stand at 37 °C for 30 min and at room temperature for a further 30 min. Lectin activity was expressed as hemagglutinating unit per milligram of protein (HU/mg of protein), with 1 HU defined as the reciprocal of the highest dilution giving a visible agglutination to the naked eye.

Nematode Egg Extraction and Production of Juveniles. *M. incognita* (race 1) eggs were extracted from infected tomato roots.[33] These roots were washed with distilled water, cut into 5 cm pieces, and ground in a blender with 0.5% sodium hypochlorite, and the eggs were collected on a 500 mesh sieve (26 μm). Second-stage juveniles (J2s) were hatched from the eggs in distilled water at approximately 28 °C for 48 h. The J2 population was counted and used as the inoculum within 1−2 days of collection.

Attraction Assay. The attraction of *M. incognita* to dialyzed soybean seed exudates was performed following a protocol based on Wang et al.[34] A total of 3 mL of 23% pluronic gel containing 800 freshly hatched J2s was pipetted onto 35 × 10 mm Falcon Easy-Grip Petri dishes (BD Biosciences, San Jose, CA) and kept at room temperature for 24 h. Thereafter, filter paper discs (6 mm diameter) soaked with seed exudates (500 and 1000 mg of protein/L) were added to the center of each dish and then kept at room temperature. The number of nematodes touching the terminal 2 mm of the disc tip was counted at 1, 3, 5, and 24 h time intervals. Distilled water was used as the negative control. The experiment was repeated twice and carried out in triplicate.

***In Vitro* Egg Hatching Test.** Egg suspensions (approximately 500 eggs in distilled water) were incubated at 28 °C in a 12 well culture plate (BD Biosciences, San Jose, CA) with dialyzed seed exudates at a final concentration of 250 and 500 mg of protein/L. The experiment was repeated twice and carried out in triplicate using distilled water as the control. After the incubation time, the number of unhatched eggs was counted under a microscope and the hatch rates were assessed 24 and 48 h after treatment.[35]

***In Vitro* Mortality/Mobility Test of J2s.** A suspension of 50 freshly hatched J2s in distilled water was incubated at 28 °C in a 12-well culture plate with dialyzed seed exudates at final concentrations ranging from 60 to 500 mg of protein/L in a final volume of 0.0005 L. Distilled water was used as the control. After 24 and 48 h of treatment, the mobile and non-mobile J2s were counted under a stereomicroscope. Nematodes were considered dead if they did not move even after the dilution of the exudates to an innocuous concentration.[36] The data were transformed into the percentage mortality [mortality (%) = dead number of J2s/total number of J2s × 100] before the statistical calculations. The experiment was repeated twice and carried out in triplicate.

Root Infection Assay. J2s were kept in contact with the dialyzed seed exudates (125 mg of protein/L) at 28 °C in the dark for 24 h. Thereafter, treated J2s were used in the root inoculation of 1-month-old tobacco seedlings growing in sand/sandy loam (2:1, v/v) in plastic pots. Each seedling received 150 J2s. Tobacco seedlings with J2s exposed to distilled water served as a control. Seedlings inoculated with J2s were maintained in the greenhouse at 28 °C and under a 12/12 h light/dark cycle. At 6 weeks after inoculation, the whole plant was collected and the root system was washed with tap water to remove the soil. The root gall numbers and egg masses were assessed.[35] Seedlings were arranged in a completely randomized block design with 10 individuals per block and three biological replicates.

Statistical Analysis. The experimental design used in all biological assays was completely randomized. The mean of each treatment was compared to its respective control. The data analysis was performed by two ways: (i) when the data were in normal distribution, the analysis was performed using analysis of variation (ANOVA) and Tukey's test to compare means ($p < 0.05$), and (ii) when the data were not in normal distribution, analysis was performed in the JMP 5.1 software using the Wilcoxon/Kruskal−Wallis non-parametric test with a

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significance level of 5%. The measurement of dispersion was calculated using standard error.

RESULTS

Protein Content and Molecular Mass of the Exuded Proteins. The type of proteins accumulated in the medium during the imbibition of soybean seeds did not change over time, because the exudates showed similar electrophoretic patterns for the different time periods analyzed. However, the highest amount of protein in the medium was observed 18 h after soaking, which amounted to approximately 3190.00 ± 100.00 mg/L, corresponding to 0.39 ± 0.01 mg/seed. Thus, the exudates obtained under this condition were selected for use in further analyses. Phytochemical compounds were not observed in the dialyzed exudates. SDS−PAGE showed that these exudates contained several proteins ranging in size from 4202 to 97 000 Da, with predominant bands of apparent molecular masses of approximately 28 000 and 45 000 Da (Figure 1).

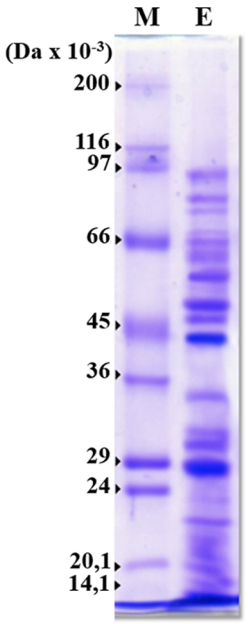

Figure 1. SDS−PAGE of soybean seed exudates: (M) mass markers and (E) soybean seed exudates collected 18 h after soaking in 0.1 M sodium acetate buffer at pH 5.0 and 28 °C. Proteins (25 μg) from seed exudates were loaded in lane E. Protein bands were stained with Coomassie Brilliant Blue G-250.

Identification and Functional Classification of the Exuded Proteins. A gel-free proteomic approach identified 63 protein accessions based on the sequences in the UniProtKB database (Table 1). The distribution of the identified proteins according to their theoretical molecular weights by ESI−LC−MS/MS was corroborated by the results of SDS−PAGE. Among the identified proteins, glycinins, lipoxygenases, proteinase inhibitors, and seed maturation proteins were the most common. Gene ontology annotation allowed for classification of the identified proteins into nine categories: nutrient reservoir activity (14 proteins), DNA binding (1 protein), binding (1 protein), catalytic activity (7 proteins), structural molecule activity (1 protein), hydrolase activity (3 proteins), response to stress (3 proteins), enzyme regulator activity (10 proteins), and carbohydrate binding (1 protein). The 22 remaining proteins were not classified. Interestingly, several proteins identified by ESI−LC−MS/MS are related to plant defense, including β-1,3-glucanase, chitinase class I, lectin, Kunitz and Bowman−Birk trypsin inhibitors, lipoxygenases, late embryogenesis abundant protein, and 7S globulin. In addition, it was observed that most proteins were secreted into the medium via a classical mechanism. However, some of them seem to be secreted via a non-classical, novel fashion pathway (Table 2), because they have scores higher than the threshold value of neural network (NN) > 0.5. One example was the Bowman−Birk protease inhibitor, which was the most highly represented protein.

Activity of Plant Defense-Related Proteins in the Exudates. Specific assays revealed the presence of activities related to some classical defense proteins in the exudates (Table 3). The presence of lectin was evaluated on the basis of the hemagglutinating activity, which was the most pronounced in the seed exudates, particularly when the trypsin-treated rabbit erythrocytes (609.52 HU/mg of protein) were used, reaching up to 4.8 times the value observed for untreated red cells (126.98 HU/mg of protein). To a lesser extent, both β-1,3-glucanase (58.26 nkat/mg of protein) and papain inhibitor (30.48 IU/mg of protein) showed high activities. On the other hand, chitinase activity was not detected, probably because of the low concentration of this protein in the soybean seed exudates.

Biological Effects of Exuded Proteins on Nematodes. A chemotaxis assay showed that the exudates have a repellent effect on the nematode (Figure 2). Indeed, the percentages of J2s of *M. incognita* touching the disc tips immersed in the exudates at 1000 mg of protein/L concentration but not 500 mg of protein/L were significantly ($p < 0.05$) lower compared to the control. This result was particularly true for 1, 3, and 5 h of exposure time. However, at 24 h of exposure, there was no difference between the control and the exudate-treated nematodes.

As demonstrated by the *in vitro* assays, the dialyzed soybean exudates also showed adverse effects on several developmental parameters of *M. incognita*. Indeed, the exudates (500 mg of protein/L) reduced the hatching of the *M. incognita* eggs at 48 h of incubation compared to the control by allowing less than 22% of the eggs to hatch (Figure 3). In addition, the motility of J2s was inhibited by the soybean exudates (60−500 mg of protein/L) compared to the control (Figure 4). The percentage of inactive J2s was approximately 90%, even at the lowest protein concentration (60 mg of protein/L) used. In general, this percentage did not change with the protein concentration of the exudates or the incubation period. Further tests revealed that the exudates were lethal to *M. incognita* J2s (Figure 5). In this case, the percentage of mortality of J2s increased with the incubation period from 24 to 48 h, reaching 100% even in one of the lowest tested protein concentrations (125 mg of protein/L).

The greenhouse experiment using tobacco plants also demonstrated the efficacy of the soybean seed exudates in the *M. incognita* controls. At 6 weeks after inoculation, the pretreatment of J2s with the soybean exudates resulted in around 90 and 94% reduction of the *M. incognita* gall and egg mass number, respectively, in tobacco plants (Figure 6) compared to the control.

DISCUSSION

Plant defense mechanisms have been studied very intensively in vegetative tissues. However, little is known about the strategies

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Table 1. Proteins Identified from Soybean Seed Exudates by ESI−LC−MS/MS

protein description[a]	UniProtKB accession number (AC)	molecular weight (MW)[b]	score[c]	coverage (%)[d]
Nutrient Reservoir Activity				
basic 7S globulin	P13917	46393.21	55389.76	58.54
glycinin	P04776	55706.47	32333.35	59.33
2S albumin	P19594	18460.09	14237.19	35.86
β conglycinin β subunit	F7J077	50441.93	8815.38	39.63
7S storage protein α subunit	Q39816	27367.78	3554.79	35.27
DNA Binding				
uncharacterized protein	I1N2Y9	26078.60	138.91	6.03
Binding				
chitinase class I	Q9SDY6	34341.45	1496.48	28.33
Catalytic Activity				
lipoxygenase	B3TDK5	96726.37	3055.39	27.26
Hydrolase Activity				
endo-1,3-β-glucanase	Q6S9W0	36603.47	3032.81	34.70
Response to Stress				
seed maturation protein PM22	Q9XER5	16688.38	5253.49	50.88
protein P21	P25096	21462.86	4481.12	17.57
Enzyme Regulator Activity				
Bowman−Birk type proteinase inhibitor D II	P01064	9467.83	43752.51	72.29
Bowman−Birk type proteinase inhibitor C II	P01063	9201.38	27008.52	65.43
trypsin inhibitor A	P01070	24005.35	17231.35	42
Kunitz trypsin inhibitor 3	Q9LD16	24067.35	11070.21	21.60
Bowman−Birk proteinase inhibitor	Q9SBA9	4530.20	679.02	67.43
trypsin inhibitor B	P01071	20040.66	8585.66	21.17
Enzyme Regulator Activity				
Kunitz type trypsin inhibitor KTI1	P25272	22546.00	603.17	25.12
Carbohydrate Binding				
lectin	P05046	30928.02	5862.31	30.52
Not Classified				
24 kDa protein SC24	Q8W0V9	24569.62	15965.57	39.42
late embryogenesis abundant protein	P93165	11491.34	15633.56	54.92
napin type 2S albumin 1	Q9ZNZ4	17834.76	12450.66	43.44
hydrophobic seed protein	P24337	8358.90	10225.60	38.75
seed maturation protein PM30	Q9XET0	15097.41	7151.64	49.28
uncharacterized protein	I1K6M2	23812.87	11258.69	55.10
uncharacterized protein	C6T0L2	11141.08	8993.93	40.26
uncharacterized protein	I1NGF5	47229.38	8818.34	44.76
uncharacterized protein	I1NGG4	26058.11	2406.17	36.72
dehydrin	Q70EL8	23733.70	1902.39	45.72
P24 oleosin isoform A	P29530	23501.69	1396.25	16.81
seed biotin containing protein SBP65	Q39846	67878.09	616.55	23.74

[a]All proteins were identified in *G. max*. [b]Theoretical molecular mass in daltons. [c]Scores were obtained using an algorithm that includes retention time, ion intensities, charge state, and accurate masses of both precursor and product ions from LC−MS data.[50] [d]Coverage means the percentage (%) of the sequence of the protein represented by the peptides identified in the MS run.

Table 2. Prediction of Non-classical Protein Secretion by SecretomeP

protein description[a]	UniProtKB AC	score[b]
Bowman−Birk type proteinase inhibitor D II	P01064	0.858
uncharacterized protein	C6T0L2	0.778
uncharacterized protein	I1L957	0.521
seed maturation protein	Q9S7N8	0.891
Bowman−Birk proteinase inhibitor	Q9SBA9	0.648
Bowman−Birk type proteinase inhibitor C II	P01063	0.913

[a]All proteins were identified in *G. max*. [b]Neural network (NN) scores of >0.5 are considered to predict extracellular localization.

Table 3. Activities of the Plant Defense-Related Proteins Present in the Soybean Seed Exudates[a]

protein activity	seed exudates
lectin (HU/mg of protein)	
untreated erythrocytes	126.98 ± 4.39
trypsin-treated erythrocytes	609.52 ± 0.01
trypsin inhibitor (mg of TI/mg of protein)	4.81 ± 0.23
papain inhibitor (IU/mg of protein)	30.48 ± 1.83
protease (AU mg^{-1} of protein min^{-1})	1.52 ± 0.05
peroxidase (AU mg^{-1} of protein min^{-1})	0.01 ± 0.00
β-1,3-glucanase (nkat/mg of protein)	58.26 ± 3.02
chitinase (nkat/mg of protein)	ND[b]

[a]Values are the means ± standard deviation (SD) of biological triplicates. [b]ND = not detected.

used by seeds to survive and germinate in substrates densely populated with microorganisms.[37] It is known that the initial infections of seeds and roots by pathogens may be driven by attractive nutritive exudates, which may be hampered by exuded defense-related compounds.[9] Thus, in an attempt to gain better insight into the role of seed exudates in plant defense, the present study focused on the characterization of exuded soybean seed proteins and their effects against the root-knot nematode *M. incognita*.

Proteins with potential roles in plant defense were identified by ESI−LC−MS/MS in soybean seed exudates. Among them, glycinin, lipoxygenase, and protease inhibitors were the predominant exuded proteins (Table 1). Indeed, several storage proteins, of which a majority are globulins, have been identified in soybeans. The plant globulin family can be subdivided into two main classes according to their sedimentation coefficients: glycinin (11S) and β-conglycinin (7S). In addition to their high nutritional value, it has been shown that these proteins exhibit antimicrobial activities against pathogenic bacteria in equivalent or even higher levels than penicillin, suggesting the involvement of these molecules in the innate defense system of soybeans.[38] Along with the traditionally classified storage proteins, plant lipoxygenases are commonly associated with plant defense

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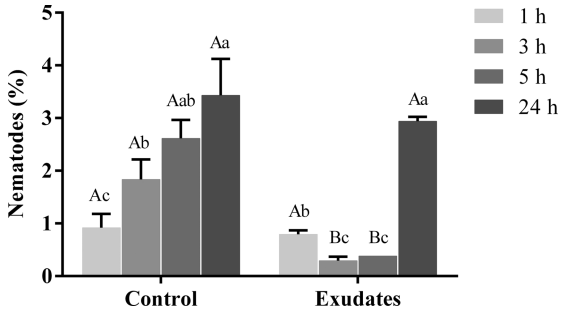

Figure 2. Chemotactic response of the *M. incognita* J2s at 1, 3, 5, and 24 h of exposure to the soybean seed exudates (1000 mg/L). Values represent the means ± SD of triplicates. Significant differences ($p < 0.05$) of the means within the control or within the exudate-treated J2s at different time points are denoted by different lowercase letters. Significant differences ($p < 0.05$) of the means comparing the controls and the exudate-treated J2s at a given time point are denoted by different capital letters ($p < 0.05$).

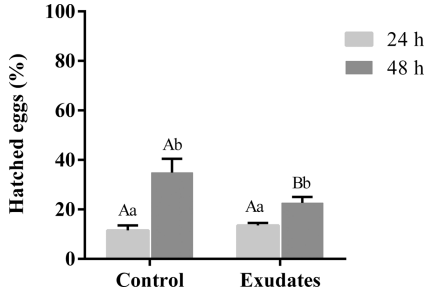

Figure 3. Effect of soybean seed exudates on *M. incognita* eggs at 24 and 48 h of exposure time. Values represent the means ± SD of triplicates. Significant differences ($p < 0.05$) of the means within the control or within the exudate-treated eggs at different time points are denoted by different lowercase letters. Significant differences ($p < 0.05$) of the means comparing the controls and the exudate-treated eggs at a given time point are denoted by different capital letters ($p < 0.05$).

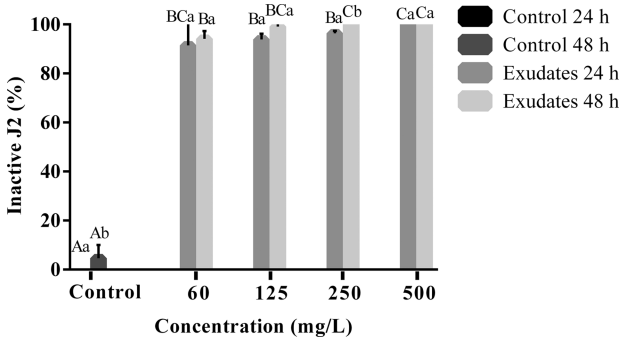

Figure 4. Inactivation of *M. incognita* J2s at 24 and 48 h of exposure to soybean seed exudates. The values represent the means ± SD of triplicates. Significant differences ($p < 0.05$) of the means within the control or within the exudate-treated J2s at different time points are denoted by different lowercase letters. Significant differences ($p < 0.05$) in the means comparing the controls and the exudate-treated J2s at a given time point are denoted by different capital letters ($p < 0.05$).

mechanisms because they are related to the synthesis of a number of different compounds with signaling functions and antimicrobial activity or the development of the hypersensitive response.[39]

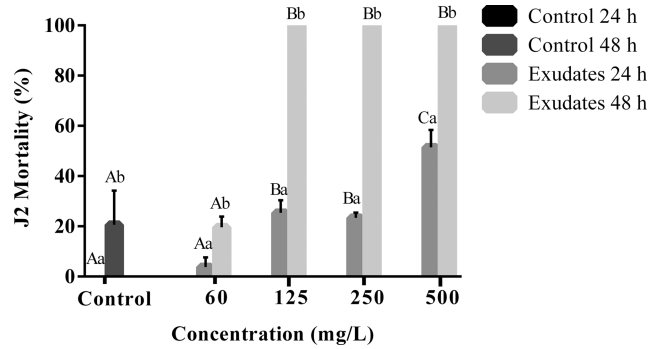

Figure 5. Mortality of *M. incognita* J2s at 24 and 48 h exposure time to soybean seed exudates. The values represent the means ± SD of triplicates. Significant differences ($p < 0.05$) of the means within the control or within the exudate-treated J2s at different time points are denoted by different lowercase letters. Significant differences ($p < 0.05$) of the means comparing the controls and the exudate-treated J2s at a given time point are denoted by different capital letters ($p < 0.05$).

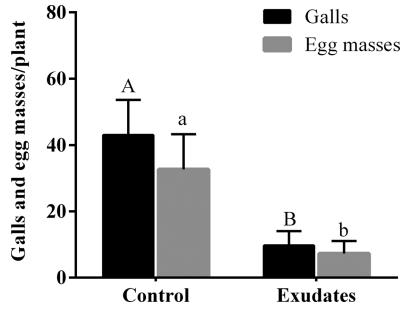

Figure 6. Effect of soybean seed exudates on *M. incognita* virulence. Gall number and egg masses were assessed 6 weeks after inoculation of tobacco roots under greenhouse conditions. Significant differences ($p < 0.05$) of means are denoted by different letters (one-way ANOVA and Tukey test).

Additionally, protease inhibitors can act as storage proteins involved in plant defense. In soybeans, trypsin inhibitors have been reported, which comprise approximately 6% of the total seed proteins. Members of the trypsin inhibitor family inhibit various proteases from plant pathogenic microorganisms and phytophagous insects, suggesting the importance of exudates in the successful seed germination.[40] Other classes of proteins, such as chitinase, β-1,3-glucanase, and lectin, were also released by imbibed soybean seeds. These results agree with those described for legume seed exudates, confirming the complexity of legume proteomes.[9,12]

Most of the exuded soybean proteins appeared to have been released into the germinating medium via a classical mechanism. In contrast, the Bowman−Birk protease inhibitor has been predicted by SecretomeP software to be potentially secreted via a non-conventional pathway. As previously noted, many proteins in soybean exudates undergo secretion via classical mechanisms, with signal peptides targeting the molecules to storage vacuoles.[41] Nevertheless, there is evidence for the existence of plant proteins that are secreted via rough endoplasmic reticulum (RER)−Golgi apparatus-independent routes. The existence of an alternative secretory route can be explained by the need for a rapid and effective defense response to external stress.[42] Accordingly, the Bowman−Birk protease inhibitor might participate in this plant defense process.

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To validate the presence of some proteins identified using the gel-free quantitative proteomic approach, the enzymatic and hemagglutinating activities were detected in the soybean exudates. Using these approaches, the presence of proteins, such as lectin, trypsin inhibitor, and β-1,3-glucanase, was confirmed. However, the use of proteomics to identify a certain protein in a sample does not always mean that the *in vitro* assay method is robust enough to detect the activity of that protein, particularly when it is present in low amounts. This can be exemplified by the absence of chitinase activity in the seed exudates, despite the detection of a class I chitinase by ESI−LC−MS/MS. Moreover, the reverse situation was also observed, in which the *in vitro* activity was detected but the corresponding protein was not identified by MS/MS. For instance, our study showed the inhibitory activity of papain in the soybean exudates *in vitro* (Table 3), although the MS/MS analysis did not identify any phytocystatins. Nevertheless, some Kunitz trypsin inhibitors have been reported as bifunctional inhibitors with the ability to inhibit two different enzyme classes (serine and cysteine proteinases).[43] Thus, it is possible that the *in vitro* papain inhibitory activity found in the soybean exudates (Table 3) might be associated with the Kunitz trypsin inhibitors identified through ESI−LC−MS/MS (Table 1).

The *in vitro* inhibitory effect of seed-exuded proteins on the fungal growth has previously been shown.[8] Nonetheless, there have been very few similar studies to date that have addressed plant parasite nematodes. Therefore, after validation of the identities of the exuded proteins involved in plant defense and their corresponding *in vitro* enzymatic activities, our next step was to assess the potential activity of the soybean exudates against *M. incognita*. Indeed, a significant reduction in the mobility and an increase in the mortality of *M. incognita* J2s were observed after treatment with the soybean exudates even at low protein concentrations (60 and 125 mg of protein/L, respectively) (Figures 4 and 5). The nematostatic and nematicidal effects of the exudates on *M. incognita* J2s were also verified using a proteinaceous papain inhibitor purified from *Crotalaria pallida* roots.[7] These effects were found to be lethal, with 95% mortality, when the parasite was exposed to a 50 μg dose of this inhibitor for 48 h.[7] Although the repellent effect of the soybean exudates toward the nematodes was transient (Figure 2), this delay affected their infectivity. This assumption is supported by our experimental findings in which tobacco plants that were inoculated with soybean seed exudate-treated J2s showed around 90 and 94% reductions in the number of galls and egg masses, respectively. The nematicidal activity of the soybean seed exudates is promising because it apparently exerted this effect during more than one stage of the life cycle of the parasite. After 48 h of treatment, the exuded proteins (at 500 mg of protein/L) inhibited the hatching of the nematode eggs by 40% (Figure 3) relative to the controls. This observation is very important because the nematode egg shell has three protective layers, which might complicate the use of chemical agents to control the further development of the nematodes.

Among the various proteins and activities detected in soybean exudates, some can be associated with the nematicidal activity against *M. incognita*, such as the papain inhibitor, which could have impaired the growth rate and fecundity of the nematode. For example, transgenic tomato overexpressing a gene that encodes for the *Colocasia esculenta* cysteine proteinase inhibitor exhibited enhanced resistance to *M. incognita* infection by means of a diminished gall number, decreased proportion of female nematodes, and reduced egg masses.[44] These effects appear to be related, at least in part, to the inhibition of the cysteine proteinase activity present in the nematode intestine.[7] In addition, the hydrolytic enzymes chitinase and protease could have also contributed to the nematicidal activity of the exudates because the *M. incognita* egg shell contains chitin and proteins.[1] This supposition in reinforced by the observation that only 30% of the eggs from transgenic tomato expressing a novel chitinase gene (PjCHI-1) were able to develop to the multi-cell/J1 stage compared to more than 96% from control plants.[45]

Lectin could also be involved in the deleterious activity of the seed exudates against root-knot nematodes. The hemagglutinating properties of lectins have been associated with the protection of plants against roundworms, because these molecules have been shown to impede the penetration of the roots by nematodes, resulting in a reduction in gall development.[46] For example, it has been reported that soybean agglutinin (SBA) interacts with amphids (sensory structures located in the nematode head region) and secretions from J2s, lowering the motility of this parasite and affecting its ability to locate the plant.[47]

In addition, because peroxidase and lipoxygenase are associated with plant responses to pathogens, these proteins may have collaborated with the nematicidal activity of soybean exudates.[26,48] For instance, lipoxygenase activity has been shown to increase in soybean roots upon infection with cyst nematodes.[49]

Taken together, our data indicate that the selective secretion of proteins by soybean seeds may contribute to the defense mechanisms against *M. incognita*. In this context, the present data bring new contributions to the understanding of the physiological role of exuded proteins in the plant defense against soil pathogens and, particularly, against parasite nematodes.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) McCarter, J. P. Molecular approaches toward resistance to plant parasitic nematodes. In *Cell Biology of Plant Nematode Parasitism*; Berg, R. H., Taylor, C. G., Eds.; Springer-Verlag Press: Berlin, Germany, 2009; Plant Cell Monographs, Vol. *15*, pp 83−113.

(2) Sasser, N. J. Worldwide dissemination and importance of the root-knot nematodes *Meloidogine* spp. *J. Nematol.* **1977**, *9*, 26−29.

(3) Caillaud, M. C.; Dubreuil, G.; Quentin, M.; Perfus-Barbeoch, L.; Lecomte, P.; de Almeida-Engler, J.; Abad, P.; Rosso, M. N.; Favery, B. Root-knot nematodes manipulates plant cell functions during compatible interaction. *J. Plant Physiol.* **2008**, *165*, 104−113.

(4) Williamson, V. M.; Hussey, R. S. Nematode pathogenesis and resistance in plants. *Plant Cell* **1996**, *8*, 1735−1745.

(5) Noling, J. W.; Becker, J. O. The challenge of research and extension to define and implement alternatives to methyl bromide. *J. Nematol.* **1994**, *26*, 573−586.

DOI: 10.1021/acs.jafc.5b01109
J. Agric. Food Chem. 2015, 63, 5335−5343

(6) Papolu, P. K.; Gantasala, N. P.; Kamaraju, D.; Banakar, P.; Sreevathsa, R.; Rao, U. Utility of host delivered RNAi of two FMRF amide like peptides, *flp-14* and *flp-18*, for the management of root knot nematode, *Meloidogyne incognita*. *PLoS One* **2013**, *8*, No. e80603.
(7) Andrade, L. B. S.; Oliveira, A. S.; Ribeiro, J. K. C.; Kiyota, S.; Vasconcelos, I. M.; Oliveira, J. T. A.; Sales, M. P. Effects of a novel pathogenesis-related class 10 (PR-10) protein from *Crotalaria pallida* roots with papain inhibitory activity against root-knot nematode *Meloidogyne incognita*. *J. Agric. Food Chem.* **2010**, *58*, 4145−4152.
(8) Nelson, E. B. Exudate molecules initiating fungal responses to seed and roots. *Plant Soil* **1990**, *129*, 61−73.
(9) Scarafoni, A.; Ronchi, A.; Prinsi, B.; Espen, L.; Assante, G.; Venturini, G.; Duranti, M. The proteome of exudates from germinating *Lupinus albus* seeds is secreted through a selective dual-step process and contains proteins involved in plant defence. *FEBS J.* **2013**, *280*, 1443−1459.
(10) Morais, J. K. S.; Gomes, V. M.; Oliveira, J. T. A.; Santos, I. S.; Da Cunha, M.; Oliveira, H. D.; Oliveira, H. P.; Sousa, D. O. B.; Vasconcelos, I. M. Soybean toxin (SBTX), a protein from soybeans that inhibits the life cycle of plant and human pathogenic fungi. *J. Agric. Food Chem.* **2010**, *58*, 10356−10363.
(11) Jolliffe, N. A.; Craddock, C. P.; Frigerio, L. Pathways for protein transport to seed storage vacuoles. *Biochem. Soc. Trans.* **2005**, *33*, 1016−1018.
(12) Palavalli, M. H.; Natarajan, S. S.; Wang, T. T.; Krishnan, H. B. Imbibition of soybean seeds in warm water results in the release of copious amounts of Bowman−Birk protease inhibitor, a putative anticarcinogenic agent. *J. Agric. Food Chem.* **2012**, *60*, 3135−3143.
(13) Pro-Seed Foundation. *Licensing of Soybean Cultivars*; http://www.fundacaoprosementes.com.br/cultivares/soja (accessed Sept 27, 2010).
(14) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities for proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(15) Matos, F. J. A. *Introdução à Fitoquímica Experimental (Introduction to Experimental Phytochemistry)*, 2nd ed.; EUFC: Fortaleza, Brazil, 1999.
(16) Laemmli, U. K. Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(17) Candiano, G.; Bruschi, M.; Musante, L.; Santucci, L.; Ghiggeri, G. M.; Carnemolla, B.; Orecchia, P.; Zardi, L.; Righetti, P. G. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* **2004**, *25*, 1327−1333.
(18) Mississippi State University. *AgBase Version 2.00*; http://www.agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.pl (accessed Dec 12, 2014).
(19) Emanuelsson, O.; Nielsen, H.; Brunak, S.; von Heijne, G. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **2000**, *300*, 1005−1016.
(20) Bannai, H.; Tamada, Y.; Maruyama, O.; Nakai, K.; Miyano, S. Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics* **2002**, *18*, 298−305.
(21) Bendtsen, J. D.; Nielsen, H.; von Heijne, G.; Brunak, S. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **2004**, *340*, 783−795.
(22) Bendtsen, J. D.; Jensen, J.; Blom, N.; von Heijne, G.; Brunak, S. Feature based prediction of a non-classical and leaderless protein secretion. *Protein Eng., Des. Sel.* **2004**, *17*, 349−356.
(23) Boller, T. Biochemical analysis of chitinase and β-1,3-glucanases. In *Molecular Plant Pathology: A Practical Approach*; Gurr, S. J., McPherson, M. J., Bowles, D. J., Eds.; Oxford University Press: New York, 1992; Vol. *II*, pp 23−30.
(24) Reissig, J. L.; Strominger, J. L.; Leloir, L. F. A modified colorimetric method for the estimation of *N*-acetylamino sugars. *J. Biol. Chem.* **1955**, *217*, 959−966.
(25) Molano, J.; Duran, A.; Cabib, E. A. Rapid and sensitive assay for chitinase using tritiated chitin. *Anal. Biochem.* **1977**, *83*, 648−656.
(26) Urbanek, H.; Kuzniak-Gebarowska, E.; Herka, H. Elicitation of defense responses in bean leaves by *Botrytis cinerea* polygalacturonase. *Acta Physiol. Plant.* **1991**, *13*, 43−50.
(27) Xavier-Filho, J.; Campos, F. A. P.; Ary, M. B.; Silva, C. P.; Carvalho, M. M. M.; Macedo, M. L. R.; Lemos, F. J. A.; Grant, G. Poor correlation between the levels of proteinase inhibitors found in seeds of different cultivars of cowpea (*Vigna unguiculata*) and the resistance/susceptibility to predation by *Callosobruchus maculatus*. *J. Agric. Food Chem.* **1989**, *37*, 1139−1143.
(28) Kakade, M. L.; Simons, N.; Liener, I. E. An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean sample. *Cereal Chem.* **1969**, *46*, 518−526.
(29) Hamerstrand, G. E.; Black, L. T.; Glover, J. D. Trypsin inhibitor in soy products: Modification of the standard analysis procedure. *Cereal Chem.* **1981**, *58*, 42−45.
(30) Abe, M.; Abe, K.; Kuroda, M.; Arai, S. Corn kernel cysteine proteinase inhibitor as a novel superfamily member of plant origin. Molecular cloning and expression studies. *Eur. J. Biochem.* **1992**, *209*, 933−937.
(31) Moreira, R. A.; Perrone, J. C. Purification and partial characterization of a lectin from *Phaseolus vulgaris*. *Plant Physiol.* **1977**, *59*, 783−787.
(32) Lis, H.; Sharon, N. Soybean (*Glycine max*) agglutinin. *Methods Enzymol.* **1972**, *28*, 360−368.
(33) Hussey, R. S.; Barker, K. R. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Dis. Rep.* **1973**, *57*, 1025−1028.
(34) Wang, C.; Lower, S.; Williamson, V. M. Application of pluronic gel to the study of root-knot nematode behaviour. *Nematology* **2009**, *11*, 453−464.
(35) Campos, H. D.; Campos, V. P.; Coimbra, J. L. Effect of root exudate of *Brachiaria decumbens* and sorgoleone of *Sorghum bicolor* on development of *Meloidogyne javanica*. *Nematol. Bras.* **2006**, *30*, 59−65.
(36) Nelson, E. B. Microbial dynamics and interactions in the spermosphere. *Annu. Rev. Phytopathol.* **2004**, *42*, 271−309.
(37) Terras, F. R. G.; Eggermont, K.; Kovaleva, V.; Raikhel, N. V.; Osborn, R. W.; Kester, A.; Rees, S. B.; Torrekens, S.; Van Leuven, F.; Vanderleyden, J.; Cammue, B. P.A.; Broekaert, W. F. Small cysteine-rich antifungal proteins from radish: Their role in host defense. *Plant Cell* **1995**, *7*, 573−588.
(38) Sitohy, M. Z.; Mahgoub, S. A.; Osman, A. O. *In vitro* and *in situ* antimicrobial action and mechanism of glycinin and its basic subunit. *Int. J. Food Microbiol.* **2012**, *154*, 19−29.
(39) Porta, H.; Rocha-Sosa, M. Plant lipoxygenases, physiological and molecular features. *Plant Physiol.* **2002**, *130*, 15−21.
(40) Cândido, E. S.; Pinto, M. F.; Pelegrini, P. B.; Lima, T. B.; Silva, O. N.; Pogue, R.; Grossi-de-Sá, M. F.; Franco, O. L. Plant storage proteins with antimicrobial activity: Novel insights into plant defense mechanisms. *FASEB J.* **2011**, *25*, 3290−3305.
(41) Cheng, F.; Williamson, J. D. Is there leaderless protein secretion in plants? *Plant Signaling Behav.* **2010**, *5*, 129−131.
(42) Delaunois, B.; Jeandet, P.; Clément, C.; Baillieul, F.; Dorey, S.; Cordelier, S. Uncovering plant−pathogen crosstalk through apoplastic proteomic studies. *Front. Plant Sci.* **2014**, *5*, 249.
(43) Migliolo, L.; Oliveira, A. S.; Santos, E. A.; Franco, O. L.; Sales, M. P. Structural and mechanistic insights into a novel non-competitive Kunitz trypsin inhibitor from *Adenanthera pavonina* L. seeds with double activity toward serine- and cysteine-proteinases. *J. Mol. Graphics Modell.* **2010**, *29*, 148−156.
(44) Chan, Y.-L.; Yang, A.-H.; Chen, J.-T.; Yeh, K.-W.; Chan, M.-T. Heterologous expression of taro cystatin protects transgenic tomato against *Meloidogyne incognita* infection by means of interfering sex determination and suppressing gall formation. *Plant Cell Rep.* **2010**, *29*, 231−238.
(45) Chan, Y.-L.; Cai, D.; Taylor, P. W. J.; Chan, M.-T.; Yeh, K. W. Adverse effect of the chitinolytic enzyme PjCHI-1 in transgenic tomato on egg mass production and embryonic development of *Meloidogyne incognita*. *Plant Pathol.* **2010**, *59*, 922−930.

DOI: 10.1021/acs.jafc.5b01109
J. Agric. Food Chem. 2015, 63, 5335−5343

(46) Marban-Mendonza, N.; Jeyaprakash, A.; Janson, H. B.; Damon, R. A., Jr.; Zuckerman, B. M. Control of root-knot nematodes on tomato by lectins. *J. Nematol.* **1987**, *19*, 31−335.
(47) McClure, M. A.; Stynes, B. S. Lectin binding sites on the amphidial exudates of *Meloidogyne*. *J. Nematol.* **1988**, *20*, 321−326.
(48) Loiseau, J.; Vu, B. L.; Macherel, M.-H.; Le Deunff, Y. Seed lipoxygenases: Occurrence and functions. *Seed Sci. Res.* **2001**, *11*, 199−211.
(49) Alkharouf, N. W.; Klink, V. P.; Chouikha, I. B.; Beard, H. S.; MacDonald, M. H.; Meyer, S.; Knap, H. T.; Khan, R.; Matthews, B. F. Timecourse microarray analyses reveal global changes in gene expression of susceptible *Glycine max* (soybean) roots during infection by *Heterodera glycines* (soybean cyst nematode). *Planta* **2006**, *224*, 838−852.
(50) Li, G. Z.; Vissers, J. P. C.; Silva, J. C.; Golick, D.; Gorenstein, M. V.; Geromanos, S. J. Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. *Proteomics* **2009**, *9*, 1696−1719.

DOI: 10.1021/acs.jafc.5b01109
J. Agric. Food Chem. 2015, 63, 5335−5343