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Application Article

Simultaneous analysis of defense-related phytohormones in *Arabidopsis thaliana* responding to fungal infection[1]

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- *Premise of the study:* Simultaneous analysis of defense-related phytohormones can provide insights into underlying biochemical interactions. Ultra-high-performance liquid chromatographic (UHPLC) techniques hyphenated to electrospray ionization mass spectrometry (ESI-MS) are powerful analytical platforms, suitable for quantitative profiling of multiple classes of metabolites.
- *Methods:* An efficient and simplified extraction method was designed followed by reverse-phase UHPLC for separation of seven phytohormones: salicylic acid, methyl salicylate, jasmonic acid, methyl jasmonate, absiscic acid, indole acetic acid, and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid. A triple quadrupole multiple reaction monitoring (MRM) method was developed for MS quantification. The methods were applied to analyze phytohormones in *Arabidopsis* leaf tissue responding to biotic stresses.
- *Results:* Under the optimized conditions, the phytohormones were separated within 15 min, with good linearities and high sensitivity. Repeatable results were obtained, with the limits of detection and quantification around 0.01 ng/μL (~9 ng/g tissue). The method was validated and applied to monitor, quantify, and compare the temporal changes of the phytohormones under biotic stress.
- *Discussion:* Quantitative changes indicate increased production of defense phytohormones from the various classes. The analytical method was useful and suitable to distinguish distinctive variations in the phytohormonal profiles and balance in *A. thaliana* leaves resulting from pathogen attack.

Key words: *Arabidopsis thaliana*; pathogen infection; phytohormones; quantitative analysis.

Plants interact with other organisms in their immediate environment where they interrelate and challenge each other. Plants are capable of resourcefully defending themselves against most pathogens because they possess innate immunity and a collection of defense processes to deter attackers such as insects and pathogens (Bezemer and van Dam, 2005). The outcome of a plant–pathogen relationship relies on the ability of a pathogen to conquer these plant defenses, which often relies on resistant obstructions, inducible defense responses, or a combination of both (Wittstock and Gershenzon, 2002; Jones and Dangl, 2006).

Defense strategies carried out by the plant are divided into two important groups: constitutive, passive defenses (which are energy costly) and inducible defenses. Inducible defenses protect the plant only upon activation of the defense mechanism through pathogen attack (Dicke et al., 2003). For inducible defenses to succeed, effective recognition of the pathogen is essential and this, in turn, leads to the activation of hormone-responsive signaling cascades for the alteration of gene expression (Ito and Sakai, 2009; Pieterse et al., 2009). Phytohormones are small organic chemical messengers that carry information within and between plant cells. These are grouped into several classes, and they can restrain or support a number of variant developmental processes, both individually and in combination (Rojo et al., 2003). Although these phytohormones are present in minute concentrations, these molecules have an intense impact on physiological processes (Kępczyńska and Krol, 2012). The phytohormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) play important roles in plant stress responses through signal mediation or regulation. The significance of SA, JA, and ET as the main primary signals in local and systemic induced defense signaling has been well documented (Pieterse et al., 2009). Moreover, the way these signal molecules function in a complex network of interacting pathways is a topic of great interest (Derksen et al., 2013). The JA and SA signaling paths act antagonistically on each other and provide the plant with a mechanism to fine-tune its defense response depending on the lifestyle of the attacker (Derksen et al., 2013). On the other hand, ET has synergistic effects on the SA signaling pathway (Pieterse et al., 2009). Research performed in the past has created additional opportunities to study how SA, JA, and ET pathways are interconnected regarding the defense signaling network (Lazebnik et al., 2014). Generally, plant responses to biotrophic pathogens, which require live tissue to complete their life cycle, are regulated by the SA signaling pathway, whereas necrotrophic pathogens that degrade plant material are regulated by the ET and/or JA signaling pathways (Pieterse et al., 2009; Tarkowská et al., 2014). In addition, all hormone pathways are connected to each other via a vast and complicated network.

The high complexity and different chemical properties of the phytohormones often necessitates more than one analytical

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technique or extraction procedure. Nonpolar or volatile hormones are amenable to gas chromatography (GC) analysis, while polar hormones are suitable to liquid chromatography (LC) or, if chemically derivatized, to GC as well. Analytical separation techniques combined to a mass spectrometer (MS) detector (GC-MS and LC-MS) allow for sensitive, repeatable, and automated quantification of hormones. Combining the analytical techniques with efficient extraction methods and sample purification and concentration steps thus provides a foundation for phytohormone profiling and analysis. For this application, electrospray ionization (ESI) tandem mass spectrometry (MS/MS) has proven to be a powerful and sensitive approach, especially when used in multiple reaction monitoring (MRM) MS/MS modes for simultaneous profiling of selected hormones (Pan et al., 2008; Müller and Munné-Bosch, 2011; Balcke et al., 2012; Liu et al., 2012; Vaclavik et al., 2013; Trapp et al., 2014).

To qualitatively identify and quantify phytohormones, the plant material needs to be homogenized and extracted with suitable solvents. The crude extract might contain interfering substances from the plant matrix that require removal to obtain a sufficiently pure sample for analysis. Therefore, the choice of the correct extraction and purification methods relies not only on the analyte of interest, but also on the type of analysis and instrumentation used (Sargent, 2013).

In this study, we describe a rapid procedure for the simultaneous extraction, purification, and quantification of SA, JA, methyl salicylate (MeSA), methyl jasmonate (MeJA), indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC), and abscisic acid (ABA) from *Arabidopsis thaliana* (L.) Heynh. leaves to understand the role of these phytohormones in the signaling network involved in the plant defense response against fungal pathogens with different lifestyles. Extracts were prepared from infected *Arabidopsis* Heynh. leaves, and samples were concentrated and purified with solid-phase extraction (SPE). Separation and identification were performed by ultra-high-performance liquid chromatography (UHPLC), and quantification, based on unique identifiers, was accomplished by a developed MRM MS/MS method.

MATERIALS AND METHODS

***Chemicals and reagents*—**SA, MeSA, JA, MeJA, ACC, ABA, IAA, the internal standard prednisolone (Pred), and MS-grade formic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All standards were analytical grade quality. Ultrapure methanol (MeOH) and acetonitrile (LC/MS grade) were purchased from Romil Pure Chemistry (Cambridge, United Kingdom).

Plant material*—Arabidopsis thaliana* (Columbia ecotype) seeds were planted in germination mix soil (Culterra, Muldersdrif, South Africa) and allowed to grow to maturity with fully developed leaf rosettes. The growth conditions were kept constant with a 12 h light/12 h dark automated light cycle in a temperature-controlled growth room set to a constant temperature of 23°C, with a light intensity of 60 µmol/m²/s. Mature 4–6-wk-old plants were used in infection studies.

Pathogen growth and plant infection*—Alternaria brassicicola* and *Colletotrichum higginsianum* were grown on half-strength potato dextrose agar (PDA; Biolab, Merck, Johannesburg, South Africa) while *Botrytis cinerea* was grown on oatmeal agar in Petri dishes at 25°C. The conidia and spores were harvested from 2-wk-old cultures by agitating mycelia in sterile water containing 0.015% Tween 20 (Sigma-Aldrich) followed by filtration through two layers of Miracloth (EMD Millipore, Billerica, Massachusetts, USA). The conidia as well as spore concentration were microscopically determined with a hemocytometer and diluted to 1×10^6/mL for inoculation. The plants were inoculated by spraying (20 mL per five plants) and transferred to a growth chamber at 22°C to obtain a humid environment, followed by incubation periods for 1, 2, 3, 4, and 5 d. The symptom development on spray-inoculated leaves was assessed based on the amount of necrotic lesions or chlorosis present, and scored based on a disease severity index (DSI) utilizing a scale of 1–5, with 1 being no symptoms and 5 being 75–100% infection. The experiment was repeated three times.

***Extraction and sample clean-up steps*—**After treatment with the three pathogens (*C. higginsianum*, *B. cinerea*, and *A. brassicicola*) over the specified period, leaves were collected, pulverized in liquid nitrogen, and frozen at −80°C until required. Two hundred milligrams (200 mg) of the tissue from treated and untreated leaves for each incubation period was weighed and suspended in 1 mL of ice-cold 50% MeOH (−20°C) (Duportet et al., 2012) at a ratio of 1 : 5 (w/v) in 2-mL microcentrifuge BashingBead lysis tubes containing ceramic microbeads (Zymo Research, Irvine, California, USA). A known concentration (1.34 ng) of prednisolone as internal standard was added into the various tubes. A FastPrep FP120 (Bio101, MP Biomedicals, Santa Ana, California, USA), kept at 5°C, was used to disrupt and homogenize the suspended plant material for 3 min at high speed and oscillations. The tubes were transferred to a microcentrifuge, and the extracts were centrifuged for 15 min at 3°C and $13{,}000 \times g$. The supernatants were transferred into clean 2-mL microcentrifuge tubes while the pellets were resuspended with 500 µL of ice-cold 50% MeOH and the extraction process repeated. The supernatants were combined and applied to 3-mL Strata-X-C SPE cartridges containing 30 mg of polymeric sorbent (33 µm, 85 Å particles, 0.9–1.2 milli-equivalents/g) (Phenomenex, Torrance, California, USA) for sample clean-up and concentration (Balcke et al., 2012). The cartridges were conditioned with 1 mL of MeOH and equilibrated with 1 mL of water before sample application onto the cartridge (1.5 mL). The SPE cartridges were eluted with 0.9 mL of 100% acetonitrile to release the phytohormones, followed by a 1 mL clean-up with MeOH. All the solvents and samples applied to the cartridges were kept at ice-cold temperatures. The eluates from the cartridges were filtered through 0.22-µm filters, transferred into chromatography vials, and stored at −20°C for subsequent analyses.

As part of the method development and validation, SPE recovery was calculated comparing the amount of each phytohormone present in samples spiked before or after extraction. The samples were spiked with a final concentration of 0.1 ng/µL (150 ng), and triplicate samples were prepared in parallel. All samples were analyzed with three repeat injections, and the concentration of each analyte was determined from the standard curves. The recovery of each analyte was determined by comparing the concentration of standards in samples spiked before and after extraction.

***Ultra-high-performance liquid chromatography*—**The extracts were analyzed on a Nexera UHPLC (Shimadzu Corporation, Kyoto, Japan) fitted with a Synergi reverse-phase C18 column (2.5 µm, 2.1 × 100 mm; Phenomenex). The injection volume was 2 µL and the column oven temperature 40°C. A binary solvent system consisting of eluent A (0.1% formic acid in Milli-Q water) and eluent B (0.1% formic acid in acetonitrile) was used. A 23-min gradient at a constant flow rate of 0.4 mL/min was used for analyte separation. The conditions were: 85% of eluent A kept constant for 3 min, initiation of gradient by 70% of eluent A at 9 min, 50% of eluent A at 12 min, 5% of eluent A at 15 min kept constant for 2 min, and at 17 min brought back to 85% to flush the column. An ultraviolet (UV) fixed wavelength detector was used at 220 and 254 nm, based on the UV-absorbing properties of the hormones.

***Triple quadrupole mass spectrometry*—**Stock solutions (5 mg/mL) of the authentic standards of SA, MeSA, IAA, ABA, JA, and MeJA, as well as the internal standard prednisolone were prepared with 50% MeOH. Mixed working solutions of these hormones and internal standard over the concentration range 0.01–250 ng/µL were prepared in 50% MeOH and stored below 4°C. MS conditions for the phytohormones and internal standard were optimized by direct infusion using the ESI source of the MS.

The MS conditions (Model 8030, Shimadzu Corporation) were as follows: the interface voltage was set at 4.50 kV for both negative and positive ionization with a heat block temperature of 399°C, interface current of 2.75 µA, and desolvation temperature of 249°C. Nitrogen was used as a drying gas at 15.00 L/min flow rate and argon as a nebulizing gas at 1.50 L/min flow rate. The ion gauge vacuum was set at 1.5e-003 Pa. The collision energy (CE) was optimized for each transition using the "MRM Optimization Method tool," an integral component of LabSolutions LCMS software (Shimadzu Corporation). The tool automates the process by collecting product ion scan data and finding the optimum CE for each transition. These MRM optimal conditions are reported in Table 1.

TABLE 1. Annotation of individual phytohormones by retention time, *m/z* values, and identification by MS/MS fragmentation patterns. LC-ESI-MS/MS analyses were performed on a UHPLC system coupled to a triple quadrupole mass spectrometer operating in both negative and positive ion modes.

No.	Analyte[a]	t_R (min)	Pseudo-molecular ion[b], (*m/z*)	Fragment ions (*m/z*) observed in MRM mode, relative intensity (%)[c]	Optimal MRM transition(s)[d]	Collision potential (CE), V	Quadrupole 1 (Q1), V	Quadrupole 3 (Q3), V	Dwell time (msec)
1	ACC	0.65	$[M+H]^+$	102	56.10 (26%), 60.10 (17%), 61.10 (57%), 102.2	**102 → 56.10**	–15	–17	–23	100
						102 → 60.10	–10	–17	–23	100
2	SA	5.95	$[M-H]^-$	137	65 (8%), 93 (92%), 137	**137 → 93**	17	28	30	100
						137 → 65	31	14	25	100
3	IAA	6.31	$[M+H]^+$	175	77 (16%), 103 (12%), 130 (72%), 175	**175 → 130**	–15	–30	–22	100
						175 → 77	–44	–12	–30	100
4	ABA	8.80	$[M+H]^+$	265	173 (7%), 229 (20%), 247 (73%), 265	**265 → 247**	–7	–18	–16	100
						265 → 229	–10	–18	–24	100
5	JA	10.41	$[M-H]^-$	209	41 (6%), 59 (94%), 209	**209 → 59**	12	22	23	100
						209 → 41	40	22	13	100
6	MeSA	11.66	$[M+H]^+$	153	65 (26%), 93 (12%), 121 (62%), 153	**153 → 121**	–14	–3	–23	100
						153 → 65	–34	–29	–22	100
7	MeJA	13.15	$[M+H]^+$	224	133 (32%), 147 (28%), 151 (40%), 225	**225 → 151**	–11	–30	–27	100
						225 → 133	–13	30	–24	100
8[e]	Pred	10.35	$[M+H]^+$	361	147.15 (30%), 325.15 (16%), 343.25 (54%), 361.1	**361 → 343**	–10	–30	–16	100
						361 → 147	–23	–30	–28	100

Note: ABA = abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; IAA = indole-3-acetic acid; JA = jasmonic acid; MeJA = methyl jasmonate; MeSA = methyl salicylate; MRM = multiple reaction monitoring; Pred = prednisolone; SA = salicylic acid; t_R = retention time.

[a] The analytes are listed in the order of elution.

[b] Scan mode: – = negative; + = positive.

[c] Precursor ions are underlined (relative % intensities of the fragments calculated in relation to that of the precursor).

[d] The transitions used for quantification are in boldface.

[e] Internal standard (IS).

***Data analysis*—**The concentrations of the phytohormones in the extracts were calculated by using the intensity vs. concentration standard curves (Table 2) generated for each individual phytohormone and integrated peak areas. From obtaining the extrapolated concentrations expressed in nanograms per microliter, the final concentrations were converted to nanograms per gram fresh weight (FW) based on the amount of plant starting material (200 mg), the recovery factor and elution volume from the SPE cartridge, as well as the injection volume (2 µL) into the LC/MS instrument. The final phytohormone concentrations of each extract were then calculated (normalized) relative to the responses obtained for the internal standard.

***Method validation*—**The method was validated in terms of selectivity, linearity, concentration range, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy (Trapp et al., 2014).

Calibration curve and linearity—The calibration curves were prepared from pure standards of SA, MeSA, IAA, ABA, JA, MeJA, ACC, and Pred, where the concentration range was: 0.01, 0.05, 0.1, 1.0, 5.0, 10.0, 50.0, 100.0, and 250.0 ng/µL. All solutions were prepared in 50% MeOH by serial dilution of working solutions.

Limits of detection and quantification—The LOD and LOQ for analytical methods based on UHPLC analysis were expressed in response units (signal-to-noise levels). The LOD for each hormone was established using leaf matrix samples spiked with a low amount of standards. However, as no analyte-free matrix was available, the LOD values for SA, MeSA, IAA, ABA, JA, MeJA, ACC, and Pred were also determined in solvent (Trapp et al., 2014).

Selectivity—Method selectivity was assessed under MRM mode based on the fragment ions in comparison to the authentic standards. Relative standard deviations (RSDs) obtained from analysis of a mixture containing all seven hormones and internal standard were calculated.

Repeatability measurements—Extracts prepared from control plants (representing the matrix) were spiked with known concentrations of phytohormone standards, prepared in triplicate and with three repeat injections of each spiked extract. The obtained RSDs were used to measure precision. Mass recovery from the spiked samples was used to determine the accuracy by evaluating the difference in concentration measured to the added amount. In addition, repeatability was assessed by analyzing each hormone prepared in triplicate at three concentration levels (low, medium, and high, corresponding to 0.05, 0.10, and 1.00 ng/µL). These analyses were performed on a single day and in a short period. The RSDs of the peak areas were calculated; % error was calculated from relative errors = (actual value − measured value) / (actual value).

RESULTS

***Extraction and sample clean-up through SPE*—**Due to the divergent physico-chemical properties of the seven hormones and internal standard, extraction procedures were evaluated by analyzing extracts from leaf tissues using MeOH as solvent (Maier et al., 2010), at various concentrations (50%, 60%, 70%, 80%, and 90%). It was found that extraction solvents with an MeOH concentration of 50% are preferred because they release lower chlorophyll content from the leaves. MeOH together with deionized H_2O was reported to yield higher extraction efficiencies compared to other extraction solvents (Trapp et al., 2014). In addition, the extracts were also spiked with known concentrations of phytohormones to calculate the extraction efficiencies of the various analytes. It was concluded that 50% MeOH was to be used for extraction, to obtain both polar and medium polar metabolites (Maier et al., 2010). In addition, a cold temperature was used (Duportet et al., 2012) to minimize potential losses of volatile hormones such as MeSA and MeJA through evaporation.

Plant extracts were spiked with a low concentration (0.1 ng/µL) of individual phytohormones and extracted by following the Balcke et al. (2012) method for SPE elution. Figure 1 indicates the recovery of the spiked phytohormones from plant extracts in which no phytohormones were detected in the flow-through eluate of the samples loaded onto the SPE cartridges. Recoveries of between 85–100% of the applied phytohormones were achieved. The single-step extraction method combined with SPE concentration was found to decrease random errors leading to sample-to-sample variation and made the method more robust.

***UHPLC–MS/MS optimization and MRM transition selection*—**Under optimized conditions SA, MeSA, IAA, ABA, JA, MeJA, ACC, and Pred were well separated. Prednisolone (a synthetic glucocorticoid) was used as the internal standard instead of isotope-labeled hormones, which are costly and not readily available. A UHPLC gradient was needed to enhance the sensitivity and to lower ionization suppression. Acetonitrile and water containing 0.1% formic acid was found to achieve baseline resolution for the seven targets as well as the internal standard within 23 min with good peak shape and peak symmetry. Optimal MS ionization for MeSA, IAA, ABA, MeJA, ACC, and Pred was achieved under positive ionization, while SA and JA were better ionized in negative ionization mode. The capillary and cone voltages of the source were optimized to maximize the precursor ion signals ($[M\text{-}H]^-$) for ESI^- and $[M+H]^+$ for ESI^+). The CE was also optimized to produce characteristic product ions from the precursor ions. The optimized

TABLE 2. Calibration curve equations and other related data for quantifying phytohormones using the developed UHPLC–MRM-MS method.

	Calibration curve				
Phytohormone	Concentration range (ng/µL)	Graph equation	Correlation coefficient (r^2)	LOD (ng/µL)	LOQ (ng/µL)
JA	0.01–250	$y = -97.048x^2 + 58580x$	0.985	0.001	0.01
MeJA	0.05–250	$y = -17.03x^2 + 9923.85x$	1.00	0.05	0.05
SA	0.01–250	$y = -75.40x^2 + 40775.25x$	0.96	0.001	0.01
MeSA	0.05–250	$y = -1.6653x^2 + 24263x$	0.998	0.01	0.05
ABA	0.05–250	$y = -82.475x^2 + 118169x$	0.996	0.01	0.05
IAA	0.05–250	$y = -289.98x^2 + 251613.07x$	1.00	0.01	0.05
ACC	0.01–250	$y = -116.51x^2 + 139903x$	0.997	0.001	0.01
Pred	0.01–250	$y = -293.62x^2 + 180430x$	0.999	0.001	0.01

Note: ABA = abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; IAA = indole-3-acetic acid; JA = jasmonic acid; LOD = limits of detection; LOQ = limits of quantification; MeJA = methyl jasmonate; MeSA = methyl salicylate; Pred = prednisolone; SA = salicylic acid.

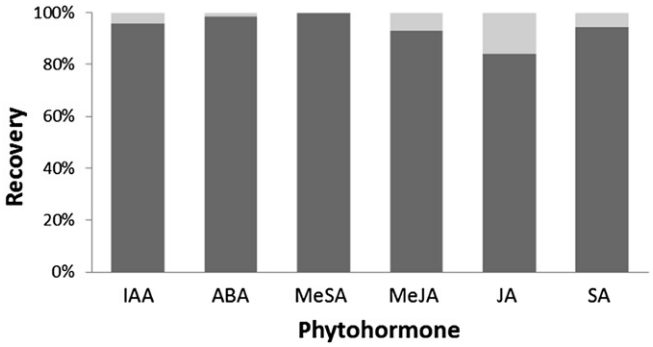

Fig. 1. Recovery of phytohormones spiked into plant extracts in 50% methanol and purified using solid-phase extraction. The light gray areas indicate the fractions found in the flow-through eluate, and the dark gray areas indicate the fractions eluted with acetonitrile. ABA = abscisic acid; IAA = indole-3-acetic acid; JA = jasmonic acid; MeJA = methyl jasmonate; MeSA = methyl salicylate; SA = salicylic acid.

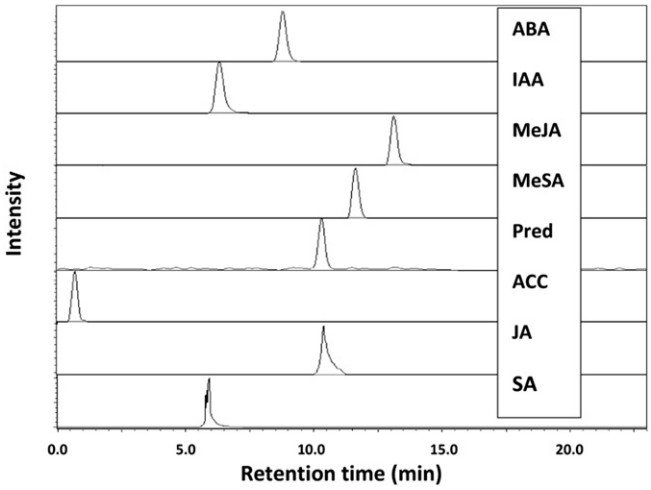

Fig. 2. Mass spectrometry chromatograms illustrating the separation of the seven targeted phytohormone standards including the internal standard prednisolone, using the optimized multiple reaction monitoring method. The chromatograms are total ion current (TIC) representations in ESI^+ mode for ABA, IAA, MeJA, MeSA, Pred, ACC, and in ESI^- mode for JA and SA. ABA = abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; ESI = electrospray ionization; IAA = indole-3-acetic acid; JA = jasmonic acid; MeJA = methyl jasmonate; MeSA = methyl salicylate; Pred = prednisolone; SA = salicylic acid.

conditions are presented in Table 1 for each of the compounds analyzed.

The different phytohormones were specifically quantified using precursor and product ions that were comparable to a previous report (Balcke et al., 2012). Using the MRM-MS method for quantification purposes of the seven phytohormones in a plant matrix permits a vital increase in sensitivity of the analytical method by reducing the noise level and contributing to the specificity of the method. For each analyte, two sets of precursor–product ion transitions were evaluated by MRM (Table 1). The first transition represents the most intense signal for the product ion and was used for quantification purposes, while the second transition was used for further confirmation of the identity of the analyte. This approach ensures that the compounds detected in the samples correspond to the peak of the standard compound detected at the same retention time. Total ion current (TIC) MS chromatograms of the seven phytohormones and prednisolone are shown in Fig. 2.

Method validation

Selectivity—Specificity (i.e., the extent to which a specific method can determine a particular analyte in a complex mixture without interference from other components; Veesman et al., 2001) was measured according to chromatographic resolution, assessing that the peaks of interest are clearly identified and separated from the rest of the peaks appearing in the plant matrix. In addition, the MS/MS spectra were assessed regarding selectivity, where the spectrums of the target analytes in the plant matrix were compared to the pure standards (Jandera, 2006; Trapp et al., 2014). The method was confirmed as selective because no additional peaks were detected on the MS chromatograms for the analytes originating from the plant matrix compared to the pure standards (Fig. 2).

Calibration curves, linearity, and limits of detection/quantification—For determining dynamic increases in phytohormone concentrations, it is essential that the calibration curves encompass the concentration of the phytohormones present in untreated plants. The range of calibration curves was defined for each compound based on previous studies (Pan et al., 2008; Balcke et al., 2012; Liu et al., 2012). Correlation coefficients (r^2) for each calibration curve, shown in Table 2, were used to evaluate the concentration : response relationship for each phytohormone and the internal standard, and thus to determine the LOD and LOQ. LOD values were determined in solvent as three times the noise level. For all the LOQ the signal-to-noise ratios were higher than 10.

Repeatability—Repeatability was evaluated to define the method's accuracy and precision by monitoring injection of various standard samples in triplicate at various known concentrations in a single day without the plant matrix. Repeatability was evaluated by comparing the mean, SD, and RSD/coefficient of variation (CV). The % error and SD for practically all standards (low, medium, and high, corresponding to 0.05, 0.10, and 1.00 ng/μL) of the phytohormones were below 15% (Appendix 1). The results thus indicate that this method is precise and accurate for quantification of the targeted phytohormones.

The repeatability of the method was validated again by monitoring injections of standard samples within the plant matrix in triplicate at various concentrations in a single day. This was conducted with three new analyte batches used for the respective calibration curves, as well as for the internal standard that was prepared and analyzed using the same extraction procedure. The CV and SDs calculated are very low (<10%) (Appendix 2).

***Pathogen infection and disease progression*—**Infection of *A. thaliana* with the four pathogens resulted in clearly discernable symptoms. A disease severity index based on the visual symptoms was compiled (Table 3) that indicates the disease progression for each pathogen infection during the five-day incubation period. Figure 3 illustrates representative chromatograms for samples extracted from leaves infected with *B. cinerea*, and Fig. 4 illustrates the quantitative data, expressed as nanograms per gram FW, obtained for all three pathogens over the five-day period.

TABLE 3. Disease severity index scores for leaf symptoms following the infection of *Arabidopsis thaliana* with three fungal pathogens over a period of five days.

	Disease rating[a]		
Day	*Alternaria brassicicola*	*Botrytis cinerea*	*Colletotrichum higginsianum*
1	1	1	1
2	1	1	1
3	3	3	2
4	4	4	3
5	5	5	4

[a] 1 = no symptoms; 2 = 1–25% infection; 3 = 26–50% infection; 4 = 51–75% infection; 5 = 76–100% infection.

DISCUSSION

A single method for the simultaneous analysis of phytohormones (with their different structural and physico-chemical properties) is difficult to achieve. For this application, MRM-MS coupled to UHPLC separation is becoming the method of choice. Key to a successful method is prior identification of the phytohormone of interest and the determination of the transitions that can be programmed into a UHPLC-MS/MS to monitor fragments of those hormones. The transition for a given hormone comprises the *m/z* values of the precursor ion and a high-intensity product ion that can be monitored at a characteristic retention time. The combination of the developed sample extraction, clean-up, chromatographic separation, and MS/MS detection and quantification methods used here was successful in creating a fast and sensitive method for analyzing and quantifying selected phytohormones important to plant defense from a small amount of leaf material. Defense-related phytohormones were prepared for quantitative analysis from an *Arabidopsis* leaf matrix through a combination of a single-step solvent extraction and solid-phase extraction clean-up and concentration. Separation conditions by gradient elution during reverse-phase UHPLC were optimized, so that each hormone exhibited a unique retention time, resulting in characteristic elution profiles that assisted in their identification. Furthermore, the MRM-MS method as described was set up to optimize the best ionization mode and ensure unique fragmentation patterns for each phytohormone. MS detector response data were normalized to that obtained for the internal standard (prednisolone, a nonplant metabolite). It was found that the UHPLC–MRM-MS method provided repeatable results and that the limit of detection and quantification (LOD/LOQ) for these phytohormones was in the region of 0.01 ng/μL (~9 ng/g tissue). The method was subsequently applied to plant samples for quantification and was successful in displaying dynamic changes in phytohormone levels of *Arabidopsis* leaves responding to pathogen infection.

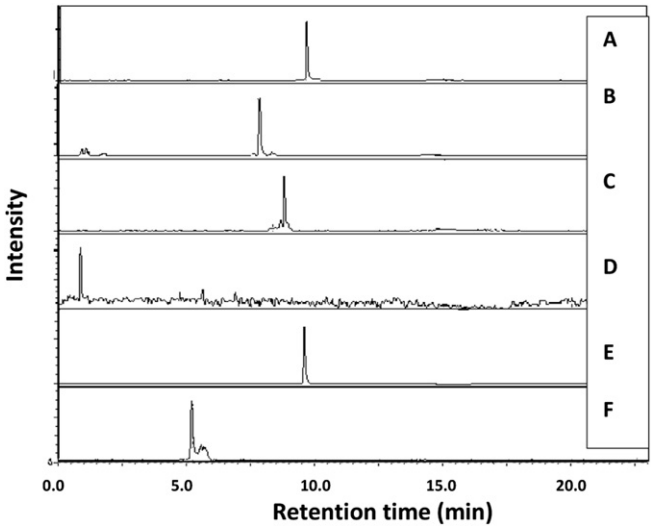

Fig. 3. UHPLC–MRM-MS chromatograms of the selected phytohormones in extracts from *Arabidopsis thaliana* leaves infected with *Botrytis cinerea*. Shown in (A–D) are Pred (A), IAA (B), ABA (C), and ACC (D) prepared from extracts at day 4 of infection, all analyzed in ESI^+ mode. Shown in (E) and (F) are JA and SA, respectively, in negative mode, present in extracts prepared at day 5 of the infection. MeSA and MeJA were not detected. ABA = abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; ESI = electrospray ionization; IAA = indole-3-acetic acid; JA = jasmonic acid; MeJA = methyl jasmonate; MeSA = methyl salicylate; Pred = prednisolone; SA = salicylic acid.

***Quantification of phytohormones in infected* Arabidopsis thaliana *leaves*—**Fluctuations of the phytohormones SA, JA (and their derivatives MeSA and MeJA), ACC (precursor of ET), ABA, and IAA in susceptible reactions were investigated. Once the UHPLC–MRM-MS method had been validated, the seven phytohormones were quantified in leaf tissue of plants responding to pathogen (necrotrophic and hemibiotrophic) infection at different time intervals after inoculation. The pattern of hormonal responses and the degree of upregulation and downregulation of phytohormonal levels are considered to be related to the type of host–pathogen relationship and type of pathogen (Spoel et al., 2007). For example, plants activate distinct defense responses depending on the lifestyle of the attacking pathogen. These responses are mainly directed by SA and JA as signaling molecules. As such, SA induces defense against biotrophic pathogens that feed and reproduce on live host cells, whereas JA activates defense against necrotrophic pathogens that kill host cells for nutrition and reproduction (De Vleesschauwer et al., 2014). Cross-talk between the different defense signaling pathways has been shown to optimize the response against a single attacker (Spoel et al., 2007).

Based on the infection by necrotrophs (*A. brassicicola* and *B. cinerea*), it was expected that JA and ACC (ET precursor) levels were to increase (Creelman and Mullet, 1995; Tuteja and Sopory, 2008). As seen in Fig. 4A and B, the leaf tissue responded to all three fungal pathogens by increasing JA levels, with the increase ranging from high (0–700 ng/g FW, *A. brassicicola*) to relatively low (40–294 ng/g FW, *B. cinerea*). The responses due to *A. brassicicola* and *B. cinerea* both portrayed a biphasic trend with an initial peak at day 2 followed by a decrease at days 3–4, before increasing strongly at day 5, the last time point of the study. In contrast, the increase due to the hemibiotroph *C. higgensianum* infection was only evident at day 5 (2430 ng/g FW).

ACC levels exhibited only minor increases and fluctuated above background levels (117–267 ng/g FW; Fig. 4C). It is uncertain to what extent ACC levels are correlated to the release of gaseous ET because it is dependent on the ACC oxidase activity and conjugation to malonyl-ACC (Kende, 1993). Responses due to *A. brassicicola* and *B. cinerea* exhibited a similar trend, with a 2.3-fold increase found on day 2 for *A. brassicicola*. In the case of *C. higginsianum*, no significant change in the levels as at day 0 was found over the time period.

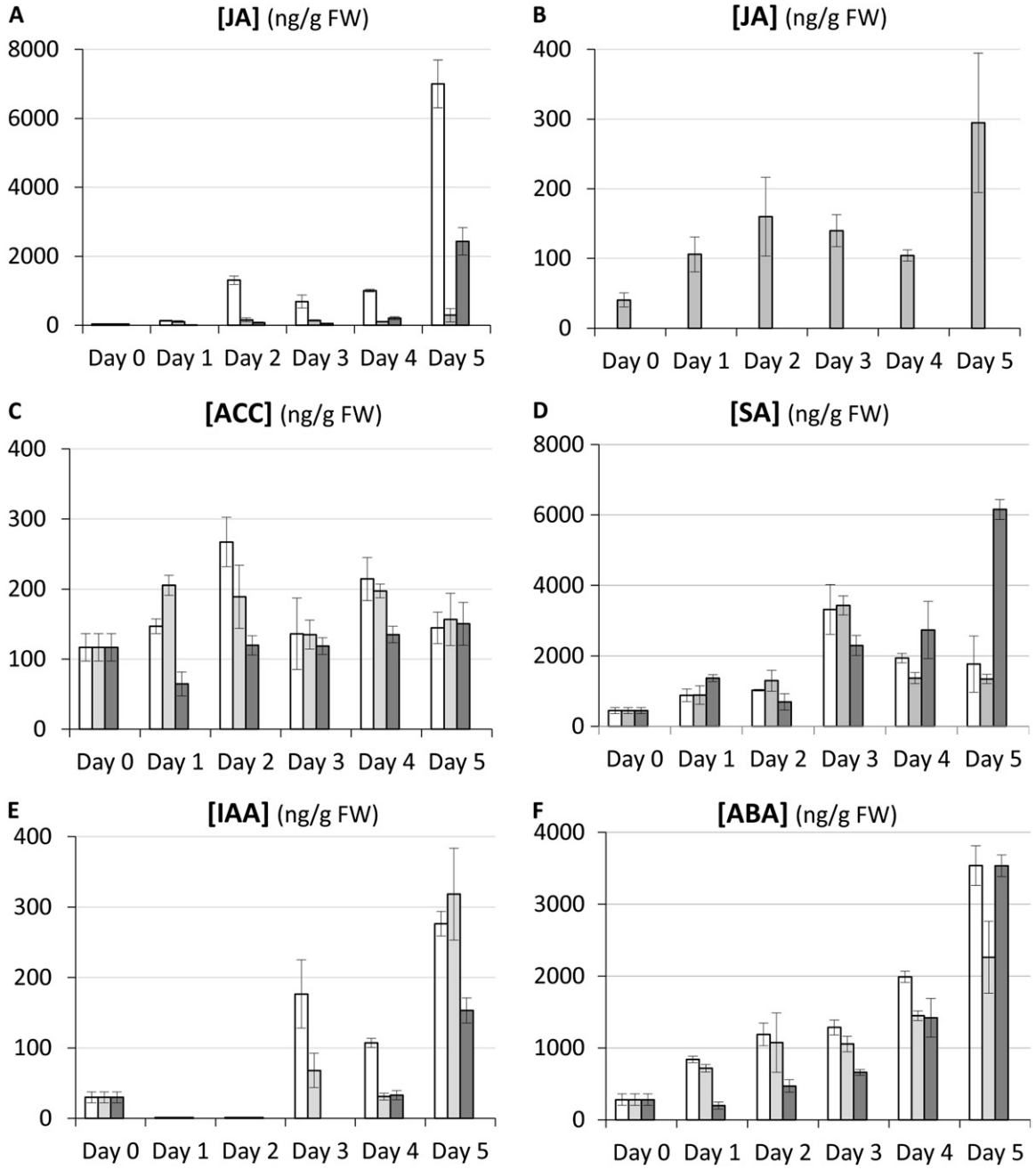

Fig. 4. Pathogen-induced changes in phytohormone concentrations in infected *Arabidopsis* leaves. Fully expanded leaves of plants (4–5 wk) were infected with the three fungal pathogens, and samples were collected at the various time intervals indicated. (A) JA (0–8000 ng/g), (B) JA (0–400 ng/g), (C) ACC (0–400 ng/g), (D) SA (0–8000 ng/g), (E) IAA (0–400 ng/g), and (F) ABA (0–4000 ng/g). Values are means ± SD (n = 3 independent samples). Extracts were prepared from 200 mg of leaf tissue, and all concentrations are expressed as ng/g fresh weight (FW). White bars = *Alternaria brassicicola*; light gray bars = *Botrytis cinerea*; dark gray bars = *Colletotrichum higginsianum.* ABA = abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; IAA = indole-3-acetic acid; JA = jasmonic acid; SA = salicylic acid.

SA concentrations can increase with two orders of magnitude during some biotrophic infections (Malamy and Klessig, 1992; De Vleesschauwer et al., 2014). Here, the increase in concentration was not as high, possibly indicative of an antagonistic effect between JA and SA (Thomma et al., 2001). Concentrations in extracts from plants infected with *A. brassicicola* or *B. cinerea* increased in parallel, peaking at day 3 (450–3400 ng/g FW). In contrast, SA concentrations in extracts from plants infected with the hemibiotroph *C. higginsianum* exhibited a consistent increase from day 1 onward, reaching a 12-fold increase (6160 ng/g FW) at day 5 (Fig. 4D).

The infections led to an apparent decrease in IAA concentrations to below the LOD on days 1 and 2, followed by a recovery and subsequent increases (0–300 ng/g FW) from day 3 onward (Fig. 4E). The decrease might be correlated to the initial redirection of metabolic flux toward the defense metabolites indole

glucosinolate and camalexin (Grubb and Abel, 2006). It has been reported that necrotroph infection with *A. brassicicola* caused increases of IAA as well as JA levels (Qi et al., 2012).

According to Adie et al. (2007), ABA has to be present for the other phytohormonal processes to occur, and relatively high concentration levels were observed for the necrotrophic pathogen infections. The results for ABA showed a steady concentration increase over the five days regardless of the pathogen, with values increasing from 250–3000 ng/g FW on average (Fig. 4F). However, these increases might be linked to a secondary response related to the desiccation of leaf tissue (Forcat et al., 2008) as the disease symptoms progressed.

MeJA is the volatile methyl ester of JA as is MeSA of SA. These hormones each activate their respective pathways in plant responses to necrotrophic and biotrophic pathogens, and are also often associated with wounding (Denance et al., 2013; De Vleesschauwer et al., 2014; Heuberger et al., 2014). The in planta concentrations of MeSA and MeJA are normally much lower than that of SA and JA (Seskar et al., 1998). Low levels of MeSA (<40 ng/g FW) were detectable from day 2 onward, but MeJA levels were below the LOD except on days 4–5. Only the extracts from *C. higginsianum*–infected plants exhibited a significant increase on day 5 where concentrations of <200 ng/g FW (MeSA) and <4 ng/g FW (MeJA) were reached (graphs not shown). The nondetection or low determined concentrations of these two analytes could also be partially due to potential losses during the SPE steps due to their volatility.

The developed method thus indicated its high sensitivity in detecting the free acids, as well as generating biologically relevant data regarding the progression of disease in infected plants. Differences in the phytohormone profiles of the three different infection studies were observed, and the results support a role for both defense pathways (JA and SA) in the response against *A. brassicicola*, *B. cinerea*, and *C. higginsianum.* The results also provide an indication that ABA is a component of the signaling network activating plant defenses during the infection period.

A plant's innate immune system is an intricate process that depends on the genetic potential to respond to attempted infection (Jones and Dangl, 2006). The complete defense response elicited by pathogens can be influenced by aspects depending on the species, developmental stage, biotic environment, and abiotic stresses. In addition, biological variation that includes the age and susceptibility of the test plants, inoculum, temperature, and humidity will also affect the results obtained. Notwithstanding, the pathogen studies validated the applicability of the simultaneous analysis of phytohormones using MRM-MS, and the current study has reaffirmed the importance of the selected phytohormones in plant defense response mechanisms due to pathogen attack.

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Appendix 1. Representative values of repeatability (accuracy and precision) obtained during the validation of the method for quantification of various phytohormones (ABA, SA, and ACC) using pure authentic standards.

	Intraday				
Phytohormone	Concentration used (ng/μL)	Peak area (mean)	Peak area (mean ± SD)	RSD (%)	Error (%)
ABA	0.05	9727	9727 ± 418	4.3	3.01
	0.10	25,721	25,721 ± 950	3.7	2.67
	1.00	430,202	430,202 ± 18,265	4.75	2.83
SA	0.05	7619	7619 ± 179	2.35	2.7
	0.10	14,395	14,395 ± 855	5.94	4.37
	1.00	144,436	144,436 ± 3901	2.7	5.12
ACC	0.05	11,279	11,279 ± 927	8.22	4.46
	0.10	32,891	32,891 ± 2176	6.62	5.94
	1.00	428,018	428,018 ± 27,444	6.41	3.85

Note: ABA = abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; RSD = relative standard deviation; SA = salicylic acid.

Appendix 2. Values of repeatability addressing accuracy and precision obtained during the validation of the method for peak quantification of selected phytohormones (SA and MeSA) and internal standard in *Arabidopsis thaliana*.[a]

Phytohormone	Concentration added (ng/μL)	Concentration obtained (mean ± SD, ng/μL)	RSD (%)	Error (%)
SA	55	52.1 ± 1.2	2.3	1.54
SA with IS	55	54.73 ± 0.15	0.28	0.21
MeSA	50	49.77 ± 0.9	1.8	1.38
MeSA with IS	50	50.6 ± 1.23	2.43	1.85
Pred (IS)	0.1	0.09 ± 0.0044	4.69	3.58
Pred in mixture	0.1	0.09 ± 0.0049	5.23	4.02

Note: IS = internal standard; MeSA = methyl salicylate; Pred = prednisolone; SA = salicylic acid.

[a] Only two phytohormones were chosen to conduct this validation step, namely SA and the related MeSA, because these compounds did not interfere with each other when analyzed in a mixture. In addition, the internal standard, prednisolone, was also included.

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