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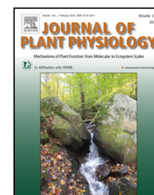

Short communication

Phytohormone profiling of the sweet orange (*Citrus sinensis* (L.) Osbeck) leaves and roots using GC–MS-based method

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ABSTRACT

Phytohormones mainly affect plant development and trigger varied responses to biotic and abiotic stresses. The sensitivity of methods used to profile phytohormones is a vital factor that affects the results. We used an improved GC–MS-based method in the selective ion-monitoring (SIM) mode to study the phytohormone profiling in citrus tissues. One extraction solvent mixture and two derivatization reagents were used, methyl chloroformate (MCF) and *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). The method showed a low limit of detection and low limit of quantification with high extraction recovery percentage and reproducibility. Overall, we detected 13 phytohormones belonging to six different groups. Auxins, SAs, *t*JA, and ABA were detected after derivatization with MCF while cytokinins and GAs were detected after derivatization with MSTFA. Cytokinins, SAs, and gibberellins were found in all tissues while auxins and *t*JA were observed only in the leaves. ABA was found in leaves and roots, but not in root tips. The method we used is efficient, precise, and appropriate to study citrus phytohormonal profiles to understand their crosstalk and responses to environmental and biological stresses.

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1. Introduction

Phytohormones are defined as a group of natural, organic molecules, and small lipophilic substances that regulate the physiological process in plants even in low concentrations (Bai et al., 2010; Bari and Jones, 2009). In the past, plant biologists were interested in only five classes of phytohormones based on their chemical structures and physiological functions, which included auxins, cytokinins, gibberellins (GAs), abscisic acid (ABA) and ethylene (ET) (Bai et al., 2010; Bari and Jones, 2009). Currently, salicylates (SAs), jasmonates (JAs), brassinosteroids (BRs), peptide hormones and strigolactones (López-Ráez et al., 2009) are also considered phytohormones.

Phytohormones play a key role in regulating developmental processes and growth, signaling networks and most physiological functions within plants including the root nodulation in leguminous plants, root growth, meristem implementation, shoot divaricating/branching, adjustment of fruit set and development, another development, and responses to biotic and abiotic stress (Quecini et al., 2007; Santner et al., 2009). Since the discovery of phytohormones, great efforts has been invested to develop and improve analytical methods for their detection including bioassay (Bai et al., 2010), ELISA (Bai et al., 2010; Rosales and Burns, 2011), and chromatographic methods which include liquid chromatography (LC) and LC–MS methods (Dobrev et al., 2005; Liu et al., 2007; Diopan et al., 2009 and Blanch et al., 2009), high performance liquid chromatography (HPLC) (Liu et al., 2007; Chiwocha et al., 2003), HPLC with tandem mass spectrometry (HPLC–MS–MS) (Chiwocha et al., 2003; Hou et al., 2008), ultra-high performance liquid chromatography (UHPLC) (Bosco et al., 2014; Floková et al., 2014) and Gas chromatography (GC) and GC–MS methods (Müller et al., 2002; Schmelz et al., 2004; Luo et al., 2013; Rawlinson et al., 2015).

Among previous methods, GC–MS was the most widely used. Generally, one compound (Rosales and Burns, 2011), one class (Ozga et al., 2009), or two or a few classes of phytohormones (Luo et al., 2013) were usually analyzed.

In this study, we aimed to analyze a large number of phytohormones belonging to six groups in citrus tissues using an improved GC–MS-based method. Studying the phytohormonal profile of citrus will allow us to better understand the balance between hormones and to study phytohormonal changes due to environmental stresses.

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2. Materials and methods

2.1. Plant materials

Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) trees were 18 months old, and 100 ± 10 cm tall when used. Trees were grown in a USDA-APHIS approved secured greenhouse under conditions ideal for citrus (28–32 °C, 16:8 L:D photoperiod, and 65% RH). For leaf sampling, three leaves were collected from each tree from top, middle and base areas and were chopped into small pieces and mixed together. For root tips, 2–3 mm from the end of the secondary roots were cut and collected together for each plant. The remainder of the secondary roots was chopped into small pieces. All samples were kept at −80 °C until extraction.

2.2. Extraction of phytohormones

Citrus tissues were ground using liquid nitrogen and 0.1 ± 0.002 g was transferred to a 1.5 mL centrifuge tube. 750 μL of the extraction solvent (methanol: water: HCl (6N); 80: 19.9: 0.1; v/v/v) was added, vortexed for 30 s, then kept on ice for 10 min. Samples were centrifuged at 15,000 rpm for 5 min at 25 °C. The supernatants were transferred to 2 mL tubes. Samples were extracted three times and the supernatants were combined then concentrated to 50 μL under a nitrogen stream and stored at −80 °C until analysis.

2.3. Derivatization of phytohormones

Acidic phytohormones, including auxins, SAs, JAs and ABA, were derivatized with MCF as described by (Hijaz and Killiny, 2014). Briefly, 50 μL of the supernatant was derivatized with 40 μL of MCF then concentrated to 20 μL under nitrogen stream and 0.5 mg of sodium sulfate were added to dry the organic phase.

For cytokinins and GAs, 50 μL from the supernatant was dried and derivatized with 100 μL of *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) by heating at 85 °C for 45 min. For GC–MS analysis, 1 μL was injected into the GC–MS running in SIM-mode.

2.4. Phytohormones standards curves

To determine the mass spectra and the retention time for each phytohormone, 1 μL of 200 ppm of derivatized standards mixture was injected into GC–MS in the full scan mode. Additionally, a 1 μL of 40, 20, 10, 5, 2.5, and 1.25 ppm of derivatized standard mixtures were injected in the SIM-mode to establish the standard curve. Typically, in the SIM-mode, three to five ions are monitored per compound and the ratios/abundances of those ions should be similar to those of the authentic standards (Luo et al., 2013; Poling and Maier, 1988; Talón et al., 1990).

2.5. Method evaluation

2.5.1. Extraction recovery

The extraction recovery (% ER) was estimated by spiking five ground samples with 5 μL of 200 ppm standard mixture then phytohormones were extracted as described above. To calculate the %ER, the detected amount of phytohormone in non-spiked samples extracted from the detected amount in the spiked sample was divided by the supplementary standard amount (20 ppm).

2.5.2. Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively. The limits were calculated using standard curves of the authentic standards.

2.5.3. Reproducibility

Sample extracts were repeatedly injected (five times) to test the reproducibility of the method. The reproducibility was estimated as relative standard deviations, RSDs (RSD = 100 × SD/mean) for retention times (relative retention times, RRTs) and peak areas (relative peak areas, RPAs) for each compound.

2.6. GC–MS analyses

We used Clarus 680 GC with SQ8-T Mass Spectrometer system (Perkin Elmer, Waltham, MA, USA) fitted with an Elite-5MS capillary column (low bleed, 30 m × 0.25 mm × 0.025 μm film thickness; Perkin Elmer, Waltham, MA, USA). Helium was the carrier gas with flow rate 1 mL min^{-1}. The temperature program for acidic phytohormones was as follows; the column was held at 50 °C for three min, and then increased to 200 °C at a rate of 4 °C min^{-1}, held for 5 min. While, the program for cytokinins and GAs was as follows; the column was held at 60 °C for 2 min and then increased to 160 °C at 20 °C min^{-1} and finally to 290 °C at 5 °C min^{-1}. The injector and the detector temperatures were set at 250 °C and 260 °C, respectively. TurboMass software version 6.1 (Perkin Elmer, Waltham, MA, USA) was used to analyze chromatograms. Identification of all phytohormones was performed by comparing their retention time, linear retention indices (LRIs), and the selected ions with those of authentic standards.

2.7. Statistical analysis

ANOVA was performed to compare the concentrations of phytohormones in different tissues. For ABA, *T*-test was applied to compare the concentrations between leaves and roots. Post hoc pairwise comparisons between treatments were performed with the Tukey honestly significant difference test.

3. Results

3.1. SAs is the most abundant phytohormone group in citrus

Benzoic (BA), *trans*-cinnamic (*t*CA) and salicylic (SA) acids were detected as salicylates (Table 1 and Fig. S1). SAs had the highest levels in all tissues. SAs were higher in leaves than roots, but they were not statistically different from root tips (Fig. 1A). BA was the highest in leaves and roots (2067.5 ng g^{-1} FW and 462.2 ng g^{-1} FW, respectively) followed by SA (672.1 ng g^{-1} FW and 193.8 ng g^{-1} FW, respectively) and *t*CA (540.3 ng g^{-1} FW and 182.9 ng g^{-1} FW, respectively) (Fig. 1A).

The SAs% ER ranged from 42.7 ± 1.7 (BA) to 61.9 ± 3.7% for *t*CA. While the LOD ranged from 0.02 ng g^{-1} FW for *t*CA to 0.04 ng g^{-1} FW for BA, the LOQ ranged from 0.08 ng g^{-1} FW to 0.12 ng g^{-1} FW. The methods showed good reproducibility (RSDs) of relative retention times (RRTs; between 0.04–0.10%) and relative peak areas (RPAs; between 3.61–5.87%) for each compound (Table 2).

3.2. Auxins are detected in citrus leaves only

Three auxins were detected in leaves (Indole-3-acetic acid (IAA), Indole-3-propionic acid (IPA), and Indole-3-butyric acid (IBA) (Fig. S1). Fig. 1B shows that auxins concentration ranged from 221.9 ng g^{-1} FW (IAA) to 258.5 ng g^{-1} FW (IPA, the highest auxin). The auxins' %ER ranged from 109.4 ± 4.7 (IAA) to 131.3 ± 3.1% (IPA) and the LOD ranged from 0.05 ng g^{-1} FW (IPA) to 0.07 ng g^{-1} FW for IBA; while LOQ ranged from 0.15 ng g^{-1} FW (IPA) to 0.22 ng g^{-1} FW for IBA. The method showed good RSDs of RRTs (between 0.049–0.098%) and RPAs (between 3.58–5.16%) for each compound (Table 2).

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