Supplementary Materials: Montmorillonite Nanoclay and Formulation with *Satureja montana* Essential Oil as a Tool to Alleviate *Xanthomonas euvesicatoria* Load on *Solanum lycopersicum*

Paulo R. Oliveira-Pinto, Nuno Mariz-Ponte, Renato L. Gil, Edite Cunha, Célia G. Amorim, Maria C. B. S. M. Montenegro, Manuel Fernandes-Ferreira, Rose M. O. F. Sousa and Conceição Santos

1. Experimental Section

1.1. Chemicals and Reagents

The 6-benzylaminopurine (BAP, ref. B0904), indole-3-acetic acid (IAA, ref. I0901), jasmonic acid (JA, ref. J0936), and methyl jasmonate (MeJA, ref. M0918) were purchased from Duchefa Biochemie. The gibberellic acid (GA, ref. G-3250) and salicylic acid (SA, ref. S-5922) were acquired from Sigma-Aldrich, whereas the (+)-abscisic acid (ABA, ref. 342401000) was acquired from ACROS Organics. The formic acid 98% (ref. 131030.1611) was obtained from Panreac AppliChem and the methanol Chromasolv (MetOH, ≥99.9%) from Honeywell. The ultra-pure water with conductivity <0.055 $\mu S\ cm^{-1}$ was produced in our laboratory (Heal force, Easy model, Shangai, China) and used whenever needed.

1.2. Mobile Phase and Standard Solutions

The two components of the mobile phase were prepared weekly. The solvents A and B were prepared by firstly diluting formic acid in ultra-pure water (0.1% (v/v)), which was then mixed with MetOH in the proportion of 60:40 (v/v) and 40:60 (v/v), respectively. Prior to use, both of the mobile phase components were degassed in an ultrasonic bath for 15 min.

The stock solutions of phytohormones were prepared in solvent A of the mobile phase (1 mg mL^{-1}) and stored at −20 °C. The dilutions of the stock solutions were prepared daily in solvent A before injection.

1.3. Analytical Method Validation

Linearity and calibration range

The linear range was assessed from the calibration curves obtained after the triplicate injection of working standard solutions of phytohormones covering different concentration ranges. Each calibration curve was established by plotting the peak area ($\mu U.A. \times s$) vs. the hormone concentration on the standard solution. Nine concentration levels were used for ABA, BAP, IAA, and SA (0.01, 0.015, 0.025, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00 $\mu g\ mL^{-1}$), for GA (0.025, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00, 2.00, and 3.00 $\mu g\ mL^{-1}$), and for JA and MeJA (0.50, 0.60, 0.75, 1.00, 5.00, 10.00, 25.00, 50.00, and 100.00 $\mu g\ mL^{-1}$). The coefficients of determination (R^2) were calculated from the intra-assay calibration curves.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of each analyte were calculated from the quotient of the standard deviation of the blank, obtained by the analysis of ten blank samples, and the slope of the calibration curve, estimated from the regression lines, multiplied by a factor of 3.3 and 10, respectively.

Precision

The precision was assessed through the repeatability and intermediate precision of the analytical signal. The repeatability was determined by triplicate analysis of the working standard solutions performed on the same day at three concentration levels for ABA, BAP, GA, IAA, and SA (0.10, 0.50, and 1.00 μg mL^{-1}) and four concentration of JA and MeJA (5.00, 25.00, 50.00, and 100.00 μg mL^{-1}). The intermediate precision was evaluated on a similar basis concerning the analysis on two consecutive days. The precision was expressed as relative standard deviation percentage (RSD%):

$$RSD\ (\%) = \frac{Standard\ deviation}{Mean}\ x\ 100\%$$

Accuracy

The accuracy was determined through the triplicate analysis of spiked samples at different fortification levels (0.10, 0.50, 0.75, and 1.00 μg mL^{-1} for ABA, BAP, GA, IAA, and SA as well as 10.00, 50.00, and 100.00 μg mL^{-1} for JA and MeJA). The results were expressed as recovery values, calculated as follows:

$$Recovery\ (\%) = \frac{Hormone_{Found} - Hormone_{Initial}}{Hormone_{Added}}\ x\ 100\%,$$

in which $Hormone_{Found}$ is the concentration of phytohormones measured in the extracts of the spiked leaf samples; $Hormone_{Initial}$ is the intrinsic concentration of phytohormones in leaf samples; and $Hormone_{Added}$ is the amount of phytohormones added to the leaf samples.

2. Results

2.1. Chromatographic separation conditions

The selection of the stationary phase and mobile phase composition was based on the UHPLC–MS method, proposed by Van Meulebroek et al. [1] to quantify phytohormones in tomato leaf samples. A reversed-phase chromatographic column and a mobile phase made by mixing aqueous formic acid and methanol were tested for the separation of seven phytohormones. Different acidified water/methanol ratios within a gradient elution program were adapted and optimized to obtain good peak resolution in the shortest run time. The optimal mobile phase is a mixture of 0.1% (v/v) formic acid in ultra-pure water and MetOH (60:40 (v/v), solvent A, and 40:60 (v/v), solvent B, respectively). The seven phytohormones were separated with a gradient elution (see section 2.7. for details) within a final run time of 30 min. Using these conditions, retention times of 6.9, 8.4, 9.8, 12.1, 15.9, 17.8, and 24.5 min were obtained for GA, BAP, IAA, SA, ABA, JA, and MeJA, respectively.

2.2. HPLC-DAD method validation:

The regression data analysis was performed to establish calibration curves for the peak area vs. hormone concentration. Linear regression lines were obtained in the range from 0.010 to 1.00 μg mL^{-1} for ABA, 0.015 to 1.00 μg mL^{-1} for IAA and SA, 0.025 to 1.00 μg mL^{-1} for BAP, 0.05 to 3.00 μg mL^{-1} for GA, and 0.60 to 100.00 μg mL^{-1} for JA and MeJA, with R^2 ranging from 0.9931 to 0.9996. The calculated values of LOD and LOQ ranged from 0.003 to 0.2 μg mL^{-1} and 0.010 to 0.6 μg mL^{-1}, respectively (Table S1).

The precision (repeatability and intermediate precision) was assessed for the working standard solutions, as depicted in Table S1. The repeatability and intermediate precision assays provided RSD% values lower than 11.1% and 13.7%, respectively, which are within the recommended limits [2], i.e., the RSD did not exceed 15%.

The recovery percentages are in agreement with the recommended limits [2] (ranging from 80% to 110% for the applied target concentrations), which proved the great accuracy of the proposed method. The mean recovery values ranged from 83.2 ± 9.9% to 109.2 ± 1.5%, with overall RSD% values lower than 10.3%. The detailed information on the recoveries and RSDs obtained for each target analyte is summarized in Table S2.

Table S1. Analytical figures of merit obtained with the proposed HPLC-DAD methodology for the quantification of phytohormones.

Validation Parameters	GA	BAP	IAA	SA	ABA	JA	MeJA
Working range, µg mL^{-1}	0.05 – 1.00	0.025 – 1.00	0.015 – 1.00	0.015 – 1.00	0.010 – 1.00	0.60-100.00	0.60-100.00
R^2	0.9980 ± 0.0013	0.9931 ± 0.0073	0.9944 ± 0.0010	0.9975 ± 0.0013	0.9996 ± 0.0002	0.9991 ± 0.0005	0.9986 ± 0.0017
Slope	29989.6 ± 356.6	86217.1 ± 4505.8	177531.1 ± 3021.4	253749.7 ± 5507.7	109267.8 ± 1381.6	7051.9 ± 5.9	7459.5 ± 75.9
Intercept	-1722.1 ± 287.5	8310.9 ± 1984.7	771.1 ± 1104.7	2965.2 ± 1021.1	1513.0 ± 472.5	-5717.4 ± 787.5	-3530.7 ± 3645.4
LOD [a], µg mL^{-1}	0.015	0.007	0.004	0.004	0.003	0.2	0.2
LOQ [a], µg mL^{-1}	0.05	0.025	0.015	0.015	0.010	0.6	0.6
Precision, RSD%							
Repeatability [b]							
0.10 µg mL^{-1}	9.4	11.1	5.0	2.8	5.3	–	–
0.50 µg mL^{-1}	4.6	5.8	2.1	2.2	4.4	–	–
1.00 µg mL^{-1}	3.4	4.9	3.3	4.0	3.6	–	–
5.00 µg mL^{-1}	–	–	–	–	–	1.1	2.8
25.00 µg mL^{-1}	–	–	–	–	–	2.1	1.1
50.00 µg mL^{-1}	–	–	–	–	–	1.9	2.0
100.00 µg mL^{-1}	–	–	–	–	–	0.2	2.6
Intermediate precision [c]							
0.10 µg mL^{-1}	5.3	12.7	4.5	3.9	1.0	–	–
0.50 µg mL^{-1}	4.6	11.8	3.6	0.3	1.2	–	–
0.75 µg mL^{-1}	4.0	3.7	1.4	0.3	3.5	–	–
5.00 µg mL^{-1}	–	–	–	–	–	13.7	13.1
25.00 µg mL^{-1}	–	–	–	–	–	1.2	1.5
50.00 µg mL^{-1}	–	–	–	–	–	2.3	0.7
100.00 µg mL^{-1}	–	–	–	–	–	0.2	0.8

[a] Based on the standard deviation of the blank and the slope; [b] *n* = 3 for each concentration level; [c] two consecutive days.

Table S2. Recovery values of the proposed methodology for the quantification of phytohormones in tomato leaf extracts (*n* = 3 for each concentration).

Analytes		Tomato Leaf		
	Added μg mL^{-1}	Found μg mL^{-1}	RSD %	Recovery %
GA				
	0.00	1.63 ± 0.01	0.8	–
	0.10	1.73 ± 0.00	0.6	96.2 ± 7.7
	0.50	2.10 ± 0.00	0.4	94.8 ± 1.5
	0.75	2.32 ± 0.01	0.5	91.6 ± 1.3
	1.00	2.58 ± 0.03	1.3	95.2 ± 2.7
6-BAP				
	0.00	N.D	–	–
	0.10	0.09 ± 0.00	4.8	105.8 ± 2.6
	0.50	0.66 ± 0.00	2.5	101.3 ± 1.9
	0.75	0.73 ± 0.01	2.0	97.6 ± 1.6
	1.00	1.06 ± 0.02	2.0	106.2 ± 1.7
IAA				
	0.00	N.D	–	–
	0.10	0.10 ± 0.00	1.9	97.7 ± 1.6
	0.50	0.54 ± 0.03	6.2	99.2 ± 5.1
	0.75	0.77 ± 0.05	6.4	102.1 ± 5.3
	1.00	1.05 ± 0.05	4.8	104.7 ± 4.1
SA				
	0.00	N.D	–	–
	0.10	0.12 ± 0.01	7.4	100.3 ± 5.8
	0.50	0.62 ± 0.01	1.8	98.1 ± 1.4
	0.75	0.82 ± 0.06	6.9	109.1 ± 6.1
	1.00	1.10 ± 0.00	0.3	110.2 ± 0.3
ABA				
	0.00	0.01 ± 0.00	4.8	–
	0.10	0.14 ± 0.01	4.1	86.1 ± 4.4
	0.50	0.58 ± 0.01	1.6	100.2 ± 1.4
	0.75	0.80 ± 0.01	0.9	104.3 ± 0.8
	1.00	1.11 ± 0.02	1.6	109.2 ± 1.5
JA				
	0.00	3.8 ± 0.6	0.9	–
	10.00	14.3 ± 0.1	0.9	104.8 ± 1.3
	50.00	52.0 ± 5.3	10.3	96.2 ± 10.7
	100.00	101.3 ± 3.1	3.1	97.5 ± 3.1
MeJA				
	0.00	7.2 ± 0.4	5.5	–
	10.00	16.0 ± 0.4	2.3	88.6 ± 3.6
	50.00	48.8 ± 5.0	10.1	83.2 ± 9.9
	100.00	104.4 ± 1.7	1.6	97.3 ± 1.7

N.D. Not detected.

References

1. Meulebroek, L.V.; Bussche, J.V.; Steppe, K.; Vanhaecke, L. Ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry for metabolomic profiling of the endogenous phytohormonal status of the tomato plant. *J. Chromatogr. A* **2012**, *1260*, 67–80. https://doi.org/10.1016/j.chroma.2012.08.047.
2. Communities, E. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.* **2002**, *221*, 8–36.