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ORIGINAL ARTICLE

A simple and efficient method for analysis of plant growth regulators: a new tool in the chest to combat recalcitrance in plant tissue culture

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Abstract This report presents a simple, rapid and accessible validated method for quantification of eight major plant growth regulators (PGR): cytokinins (6-(γ,γ-dimethylallylamino)purine (2-iP), benzylaminopurine (BA) and zeatin), auxin (indole-3-acetic acid; IAA), jasmonic acid (JA), salicylic acid (SA), gibberellic acid (GA3) and abscisic acid (ABA) by liquid chromatography mass spectrometry. This method was tested in eight species including agricultural, ornamental and medicinal species: St. John's wort, African violet, banana, American elm, tobacco, potato, sweet wormwood, and fennel. The method has good reproducibility and good sensitivity with %RSD (percent relative standard deviation) between 1 and 10% for all matrices and recovery values of 89 to 118% for all analytes. Method detection limits were 50.65 ng/g, 203.4 ng/g, 50.65, ng/g, 50.65 ng/g, 203.4 ng/g, 12.7 ng/g, 193 pg/g and 3.08 ng/g, for SA, IAA, zeatin, JA, GA3, ABA, 2-iP, and BA, respectively. Our results with a range of plant species show that this method represents a simple, low-cost method for analysis of PGRs, and may also serve as an useful starting point for the analysis of other related PGRs, as demonstrated by inclusion of the SA derivative, acetylsalicylic acid, and the JA derivatives: 12-oxo-phytodienoic acid and JA-isoleucine. The efficiency of this method will enable its incorporation into the plant tissue culture work flow and through characterization of endogenous PGR levels, will allow for improved method development for recalcitrant species facilitating fundamental and applied studies in plant morphogenesis, propagation and conservation.

Keywords Auxin · Cytokinin · Abscisic acid · Jasmonate · Salicylate · Recalcitrance

Abbreviations

ABA	Abscisic acid
ASA	Acetylsalicylic acid
BA	6-Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
GA3	Gibberellic acid
IAA	Indole-3-acetic acid
2-iP	6-(γ,γ-dimethylallylamino)purine
JA	Jasmonic acid
JA-Ile	Jasmonic acid isoleucine
MeOH	Methanol
MS	Mass spectrometry
OPDA	12-Oxo-phytodienoic acid
PCIB	p-Chlorophenoxyisobutyric acid
PGR	Plant growth regulator
%RSD	Percent relative standard deviation
SA	Salicylic acid
SIR	Single ion recording
SJW	St. John's wort
SLV	Single lab validation
TIBA	2,3,5-Triiodobenzoic acid
TDZ	Thidiazuron
UPLC	Ultra-performance liquid chromatography

Introduction

Plant growth regulators are important biomolecules in plants which exist at low concentrations and act as important

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signalling compounds mediating almost all plant processes. They are important in not only day to day physiological functions, adaptations, growth and development processes, but may respond in specialized manners to developmental or stress related cues. Auxins represent the dominant class, and to date the major active endogenous auxin has been identified as: indole-3-acetic acid (IAA). Auxins have been identified to play important roles in almost all plant processes, though are best recognized for the roles they play in apical dominance, phototropism and root growth (Enders and Strader 2015). Cytokinins including 6-(γ,γ-dimethylallylamino)purine (2-iP), benzylaminopurine (BA) and zeatin often act in close relation to auxins to mediate growth patterning (Moubayidin et al. 2009; Werner and Schmülling 2009). In particular, the balance between these two compounds was identified in the late 1950s as an important determinant of plant growth outcomes in in vitro plant culture, with cytokinins promoting shoot and lateral bud growth (Skoog and Miller 1957). Gibberellins, first identified from a fungi, are best recognized for their roles in shoot elongation and germination and gibberellic acid 3 (GA3) is the primary gibberellin utilized in vitro and in exogenous treatment of plants (Gantait et al. 2015). Salicylic (SA), jasmonic (JA) and abscisic acid (ABA) all play more specialized roles in plants being involved in many plant stress responses and their actions are often antagonistic or synergistic, particularly between SA and JA (Holopainen and Blande 2012; Huang et al. 2012; Sherif et al. 2014; Song et al. 2014).

The amount and location of plant growth regulators within a given plant are critical in determining the growth and developmental outcomes of a plant in vitro and in vivo (Enders and Strader 2015; Gantait et al. 2015; Moubayidin et al. 2009; Werner and Schmülling 2009). Thus, identification of the quantities of these compounds in a given system which is being studied can allow for explanation of observed processes as well as providing information on how best to proceed with further studies. In the case of endogenous auxin, successful regeneration of plant species in vitro is dependent on the correct balance of auxin to other PGRs, particularly cytokinins. Therefore in plants which possess high levels of endogenous auxin, it is sometimes necessary to include an auxin transport or action inhibitor such as TIBA (2,3,5-triiodobenzoic acid) or PCIB (p-chlorophenoxyisobutyric acid), respectively (Skoog and Miller 1957; Shukla et al. 2012; Jones et al. 2014). Oftentimes the necessity for inclusion of these compounds is not identified until after several unsuccessful attempts at establishing in vitro cultures, which may span over months and cost significant financial resources. By first quantifying the endogenous levels in these tissues, researchers would eliminate these costly studies by affording the investigator the insight to include appropriate combinations of growth regulators as well as their inhibitors from the outset. Further, structurally very different compounds such as a cotton defoliant (thidiazuron; TDZ), herbicides (2,4-dichlorophenoxyacetic acid; 2,4-D), growth retardants and stimulators e.g. paclobutrazol and brassinolide and many other unclassified compounds have been identified to possess plant growth-regulating properties which may be mediated by endogenous levels of plant hormones. This is particularly important for regenerating tissues that are induced with brief exposure to inductive stimulus such as TDZ or 2,4-D, and those for which organogenesis occurs on a hormone-free medium (Sanago et al. 1995; Murthy et al. 1998, 2008; Li et al. 2000; Murch and Saxena 2001; Mithila et al. 2003; Kaur and Kothari 2004).

In particular, the endogenous concentrations of phytohormones could be a valuable tool in the tissue culture kit when approaching the problem of culturing recalcitrant species. Recalcitrant species are species which, for a variety of often poorly understood factors, are resistant to establishment of in vitro tissue cultures. This represents a significant commercial and scientific problem as it hinders both investigation of the system and propagation of the plants and serves as a barrier to application of technologies such as mass multiplication or cryopreservation. Recalcitrance is, therefore, an issue for which many approaches have been taken, but which generally involve large multi-factorial studies using different application rates of a diversity of plant growth regulators, most commonly: auxins, cytokinins, and gibberellins, use of antioxidants to combat browning, desiccation and choice of varying age, tissue type or growth conditions of source materials among others (Cutler et al. 1989; Pliego-Alfaro et al. 1996; Hewezi et al. 2003; Assani et al. 2006; Mundhara and Rashid 2006; Bonga et al. 2010; Xu et al. 2013; Nguyen et al. 2016).

Analytical methods for individual classes or several classes of PGRs are available in the literature, however, often they require complex extraction methods requiring different extraction solvents and procedures for different compounds, some of which may involve hazardous solvents such as trifluoroacetic acid or ethers, or time consuming steps such as solid phase extractions or derivatization (Pan et al. 2010; Li et al. 2015; Cai et al. 2015). Additionally, these methods often put an emphasis on the use of highly specialized and complex instruments such as triple-quadrupole or time of flight systems, which represent valuable sources of in-depth chemical information or extremely high sensitivity, but which may not be readily accessible or practical for use in many laboratories without the pre-existing expertise or funds available for outsourcing of chemical analysis.

As method development from scratch can be an extremely laborious and time-consuming processes, requiring a researcher with the appropriate background, it is often advisable to start with a standardized method and modify the protocol as required for the given sample and desired analytes.

Another common difficulty in quantification of any analyte, however, is assessing the quality of a published method. Single lab validations (SLVs) as presented in this paper, provide a level of confidence for the end user in the chosen method, and eliminate the need for the end user to undergo further in depth analytical analysis to assure that the method is fit for purpose, that is, suitable for the system in which the researcher is working, namely plants (Betz et al. 2011).

Unfortunately, due to the complexity of analytical techniques and often high equipment start-up costs, quantification of PGRs in vitro is often not an option for many plant tissue culture and plant physiology labs. This paper presents an efficient method for plant growth regulator analysis which does not require in-depth analytical chemical expertise, can be performed on a relatively simple and low-cost analytical platform (Bu et al. 2016), and which does not require the use of particularly hazardous chemicals. This method provides a new approach for investigation and establishment of in vitro plant cell and tissue cultures of recalcitrant species and is complimentary to existing tissue culture approaches.

Materials and methods

Study materials

Eight species and three sample types (root, shoot and seed) were utilized for validation for a total of 12 matrices: St. John's wort (*Hypericum perforatum* L.: SJW) roots and shoots, banana (*Musa* sp.) roots and shoot, African violet (*Saintpaulia ionantha* Wendl.) shoots, potato (*Solanum tuberosum* L. cv 'Shepady') shoots, sweet wormwood (*Artemisia annua* L.; *Artemisia*) shoots and roots, tobacco (*Nicotiana tabacum* L.) shoots and roots, American elm (*Ulmus americana* L.) shoots, and fennel (*Foeniculum vulgare* Mill.) seeds. Shoots consisted of both stems and leaves. Fennel seeds were purchased from a local supermarket in Guelph, Ontario, and all other samples were taken from in vitro grown plantlets obtained from the in vitro germplasm collection at the Gosling Research Institute for the Plant Preservation (GRIPP), University of Guelph, and cultures maintained at 26 °C under a 16 h photoperiod.

Design of method validation

Accuracy of the method was evaluated by spiking samples with a known amount of each analyte to each matrix (Table 1). The method was then utilized to determine the quantity of the spike after subtracting endogenous concentrations of each analyte in the matrix and this value was then compared to the expected value. Precision was evaluated by calculating the relative standard deviation for all measurements for a particular matrix and analyte at each concentration. No fewer than nine determinations were made on three different days, with no less than 2 days separating each set of samples. Accuracy and precision were evaluated across the entire study to ensure method robustness across different days.

Instrument and method limit of detection and limits of quantification were determined according to accepted practices (AOAC 2013, Bliesner 2005), with the limit of detection set to a signal-to-noise ratio of 3:1, and the lower limit of quantitation set to a signal-to-noise of 10:1.

Table 1 Low and high spike concentrations for analytes

Analyte	Low spike concentration (µg/mL)	High spike concentration (µg/mL)
SA	0.5	5
IAA	0.5	5
JA	0.5	5
ABA	0.5	5
GA3	0.5	5
Zeatin	0.05	0.5
BA	0.05	0.5
2-iP	0.05	0.5

ABA abscisic acid, *BA* benzylaminopurine, *GA3* gibberellic acid, *IAA* indole-3-acetic acid, *2-iP* 6-(γ,γ-dimethylallylamino)purine, *JA* jasmonic acid, *SA* salicylic acid

Sample preparation

Approximately 150 mg of each tissue sample was ground in liquid nitrogen and suspended in 0.5 mL of extraction solvent composed of 50% methanol (MS Grade, Fisher Scientific, Canada; MeOH) and 4% acetic acid (glacial, Fisher Scientific, Canada) in Milli-Q water. Samples were then sonicated for 15 min on ice and spun down (2 min, 13,000 rpm) and, supernatant removed. Supernatant was then filtered through a 0.45 µm centrifuge filter (Millipore; 1 min, 13,000 rpm) and the flow through was diluted ten times in 10 mM ammonium acetate adjusted to pH 9 with ammonium hydroxide (Sigma Aldrich, Canada). Prior to analysis samples were either left unspiked or spiked with a high or low concentration of mixed standard (concentrations of spikes are given in Table 1). All standards, except jasmonic acid isoleucine (JA-Ile) and 12-oxo-phytodienoic acid (OPDA) were analytical grade and purchased from Sigma Aldrich, Canada. Standards for JA-Ile and OPDA were purchased from ChemIm Ltd (Olomouc, Czech Republic). Structures of all analytes are given in Fig. 1.

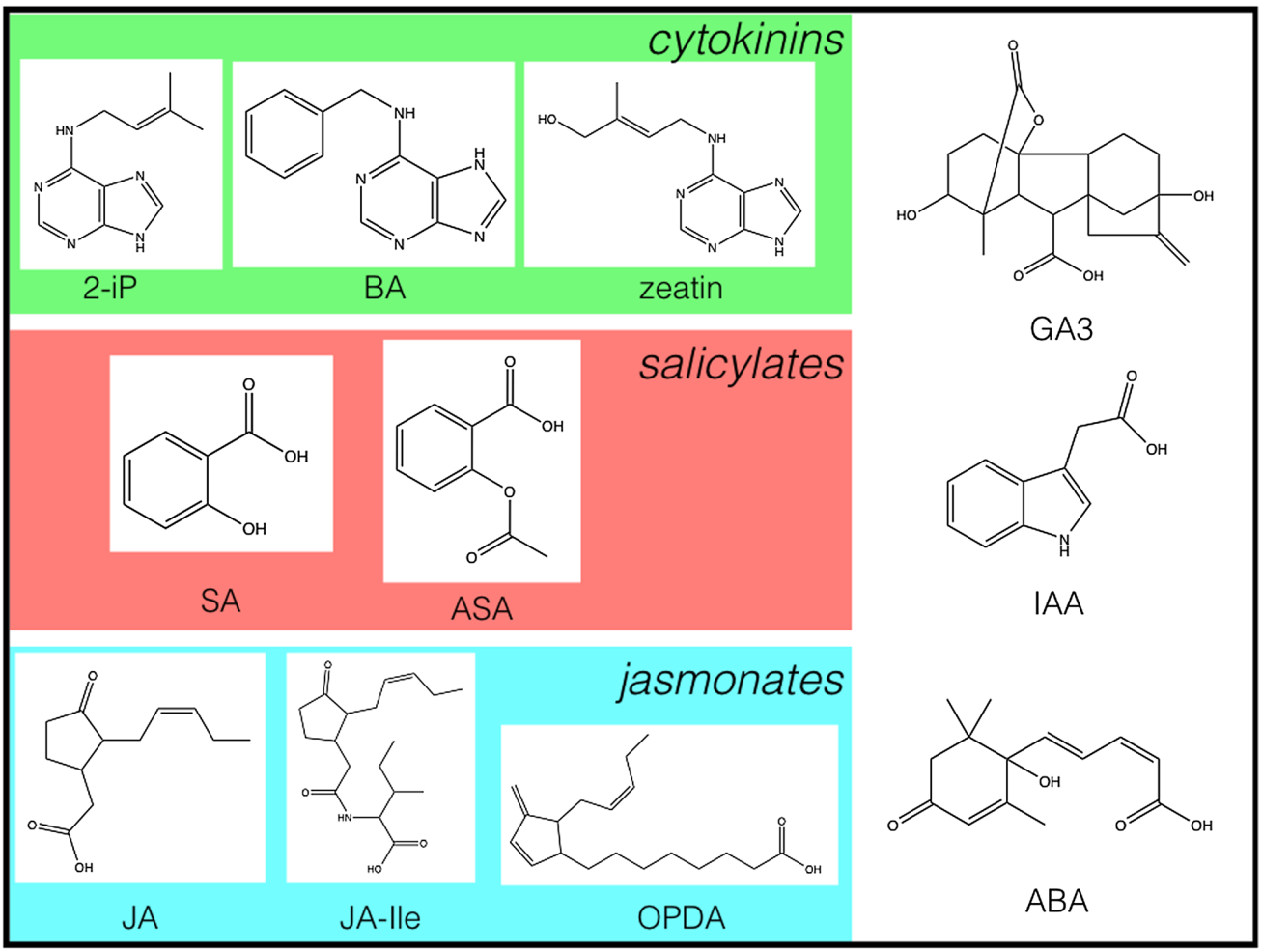

Fig. 1 Structures of analytes examined, including their derivatives which were added to the method after validation. *ABA* abscisic acid, *ASA* acetyl salicylic acid, *BA* benzylaminopurine, *GA3* gibberellic acid, *IAA* indole-3-acetic acid, *2-iP* 6-(γ,γ-dimethylallylamino)purine, *JA* jasmonic acid, *JA-Ile* jasmonic acid isoleucine, *OPDA* 12-oxophytodienoic acid, *SA* salicyclic acid [created using Keynote (Apple) and Chem Draw v15 (Perkin Elmer)]

Detection and quantification

For quantification of samples by LC-MS, 3 µL of sample was injected onto a Waters Acquity BEH Column (2.1 × 50 mm, i.d. 2.1 mm, 1.7 µm) on a Waters Acquity Classic ultra-performance liquid chromatography (UPLC) system with detection using an Aquity QDa single quadrupole mass spectrometer (MS) controlled by Empower 3 (Waters, Canada). Samples were run on a gradient with A—10 mM ammonium acetate pH 9, adjusted with ammonium hydroxide; B—100% MeOH with initial conditions of 95% A 5% B increased to 5% A 95% B over 4.5 min using a curve of 8. Column temperature was 40 °C and flow rate was 0.5 mL/min. Compounds were monitored in single ion recording (SIR) mode and quantified used standard curves (see Table 2 for MS parameters). In all cases capillary voltage was 0.8 kV, and probe temperature was 500 °C with a gain of five.

Table 2 Summary of mass to charge ratios (m/z) and ionization mode utilized for quantification of analytes in single ion reaction (SIR) mode

Analyte	m/z	Ionization mode	Cone voltage (V)
SA	137	ESI−	10
IAA	176	ESI+	10
JA	209	ESI−	15
ABA	265	ESI+	10
GA3	345	ESI−	15
Zeatin	220	ESI+	15
BA	226	ESI+	15
2-iP	204	ESI+	15

ABA abscisic acid, *BA* benzylaminopurine, *GA3* gibberellic acid, *IAA* indole-3-acetic acid, *2-iP* 6-(γ,γ-dimethylallylamino)purine, *JA* jasmonic acid, *SA* salicylic acid

Statistical analysis

All samples were prepared and analyzed in triplicate and percent relative standard deviations (%RSD) was calculated using samples run on at least three separate days, which were at least 1 day apart. All statistical analyses were performed in GraphPad Prism v6 and Microsoft Excel (Office 365).

Results

The method presented in this paper showed good *specificity* for all compounds due to the use of a single quadrupole system in SIR mode (Fig. 2), with all peaks being completely resolved from surrounding peaks and showing good signal to noise in the linear range.

Instrument *limits of detection* were 152, 610, 152, 152, 610, 38.10, 23.12 and 92.5 pg/mL for SA, IAA, zeatin, JA, GA3, ABA, 2-iP, and BA, respectively. Method detection limits were found to be 50.65 ng/g, 203.4 ng/g, 50.65, ng/g, 50.65 ng/g, 203.4 ng/g, 12.7 ng/g, 193 pg/g and 3.08 ng/g. The linear range (lower limit of quantification; LLOQ–upper limit of quantification; ULOQ) for each analyte was 6.1 ng/mL–25 µg/mL, 24.4 ng/mL–6.25 µg/mL, 6.1 ng/mL–25 µg/mL, 6.1 ng/mL–6.25 µg/mL, 24.4 ng/mL–25 µg/mL, 1.52 ng/mL–1.56 µg/mL, 193 pg/mL–1.56 µg/mL and 3.08 ng/mL–1.56 µg/mL for SA, IAA, JA, ABA, GA3, zeatin, BA and 2-iP respectively. This shows a linear range of more than four orders of magnitude for all analytes (Table 3).

Excellent *reproducibility*, presented as % RSD, was demonstrated for all eight PGRs in all of the twelves matrices, with the cytokinins BA and 2-iP showing the lowest variability. Calculated values for %RSD spanned between 1–6% for low spikes and 2–10% for high concentration for SA; from 2 to 8% and 5–6% in low and high spikes respectively for IAA; 1–8% and 2–4% for JA; 3–5% and 1–4% for ABA;

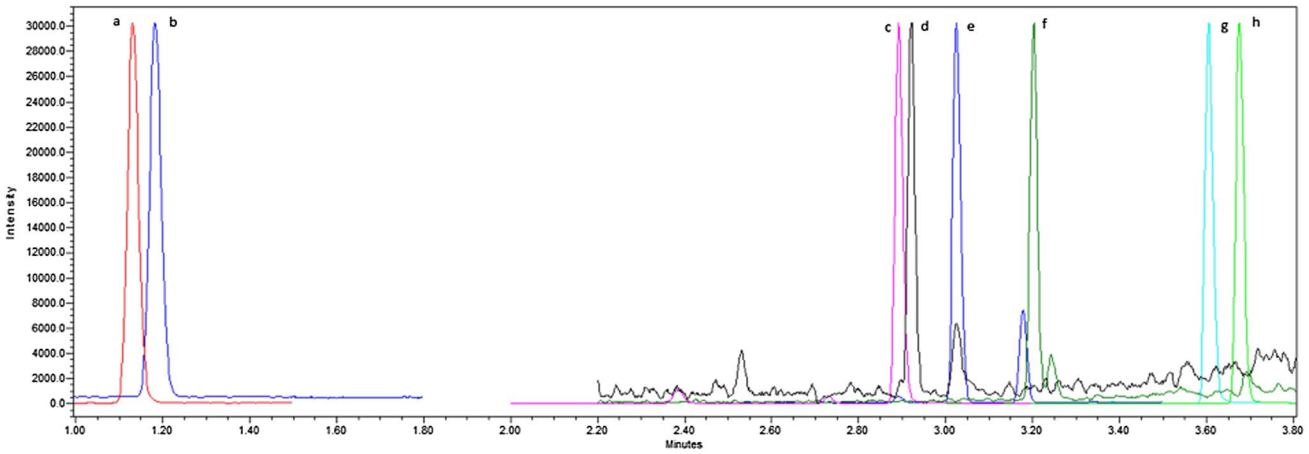

Fig. 2 Overlay of PGRs chromatograms. Salicylic acid (*a*); indole-3-acetic acid (*b*); zeatin (*c*); gibberellic acid (*d*); abscisic acid (*e*); jasmonic acid (*f*); 6-benzylaminopurine (*g*); 6-(γ,γ-dimethylallylamino) purine (*h*). *Different colours* indicate different channels (created in Empower 3, Waters). (Color figure online)

Table 3 Summary of retention time, limits of detection (LOD) and quantification (LOQ) for all analytes investigated

Analyte	Retention time (min)	Instrument LOD (pg/mL)	Method LOD (ng/g)	Instrument LLOQ (ng/mL)	Method LLOQ (ng/g)	Instrument ULOQ (µg/mL)	Method ULOQ (µg/g)
SA	1.08	152	50.65	6.1	203.3	25	833
IAA	1.13	610	203.4	24.4	810.0	6.25	208.3
JA	3.10	152	50.65	6.1	203.3	25	833
ABA	2.98	152	50.65	6.1	203.3	6.25	208.3
GA3	2.90	610	203.4	24.4	810.0	25	833
Zeatin	2.88	38.10	12.7	1.52	50.65	1.56	52
BA	3.60	23.12	0.193	0.0925	3.08	1.56	52
2-iP	3.66	92.5	3.08	0.381	12.7	1.56	52

ABA abscisic acid, *BA* benzylaminopurine, *GA3* gibberellic acid, *IAA* indole-3-acetic acid, *2-iP* 6-(γ,γ-dimethylallylamino)purine, *JA* jasmonic acid, *LLOD* lower limit of detection, *LLOQ* lower limit of quantification, *ULOQ* upper limit of quantification, *SA* salicyclic acid

2–10% and 1–8% for GA3; 1–6% and 1–3% for zeatin; 2–5% and 0–3% for BA and 1–3% and 0–4% for 2-iP (Tables 4, 5, 6, 7, 8, 9, 10, 11).

Recovery was also good for all matrices and across all of the PGRs tested. Artemisia and fennel matrices showed the greatest variability with average percent recovery differing by more than 10% in several analytes (SA, IAA, GA3, BA and 2-iP), though none was ever greater than 16%. Low concentration spike recoveries ranged from 90% in Artemisia root to 110% in fennel seed for SA; 95–118% for IAA (banana root and *Artemisia* shoot); 81–90% for JA (*Artemisia* root and fennel seed); 84–104% for ABA (SJW root and fennel seed); 88–103% for GA3, with SJW root, elm shoot and tobacco shoots all showing 84% average recovery at the low end and *Artemisia* root at the high end; 85–101% for zeatin (SJW root and fennel seed); 86–114% for BA (SJW and *Artemisia* shoot); and 91–112% for 2-iP (banana and *Artemisia* shoot).

At high concentration recoveries were similar with values of 92–111% for SA (*Artemisia* shoot and fennel seed); 89–110% for IAA (fennel seed and Artemisia root); 80–93% for JA (*Artemisia* rood and fennel seed); 97–105% for ABA with both SJW root and African violet shoot showing percent average recoveries of 97% and fennel seed being 105%; 99–111% for GA3 (tobacco shoot and *Artemisia* root); 93–107% for zeatin (SJW root and *Artemisia* root); 93–106% for BA (SJW shoot and fennel seed); and 94–116% for 2-iP (banana shoot and *Artemisia* shoot) (Tables 4, 5, 6, 7, 8, 9, 10, 11).

Inclusion of ASA into the existing method was possible with no changes to the previously described method, and could be monitored in the channel used for SA quantification. Inclusion of the jasmonates JA-Ile and OPDA were included with only minor modifications to the method. Addition of a 0.5 min hold at 95% MeOH, and addition of separate channels for mass to charge ratios specific to each

Table 4 Recovery data for salicylic acid (SA) in plant matrices at low and high spike concentrations

Species	Tissue	Low recovery average (%)	Low recovery %RSD	High recovery average (%)	High recovery %RSD
SJW	Shoot	108	2	99	4
SJW	Root	101	2	97	4
African violet	Shoot	98	2	97	4
Banana	Shoot	98	3	96	4
Banana	Root	98	3	97	4
Elm	Shoot	99	2	96	4
Tobacco	Shoot	95	3	94	4
Tobacco	Root	94	3	93	4
Potato	Shoot	96	5	93	4
Artemisia	Shoot	96	6	92	4
Artemisia	Root	90	3	99	10
Fennel	Seed	110	1	111	2

Table 5 Recovery data indole-3-acetic acid (auxin) in plant matrices at low and high spike concentrations

Species	Tissue	Low recovery average (%)	Low recovery %RSD	High recovery average (%)	High recovery %RSD
SJW	Shoot	96	5	92	6
SJW	Root	96	5	93	6
African violet	Shoot	99	5	96	6
Banana	Shoot	95	5	95	6
Banana	Root	107	8	94	6
Elm	Shoot	98	4	95	5
Tobacco	Shoot	100	5	95	6
Tobacco	Root	107	4	98	6
Potato	Shoot	110	4	102	6
Artemisia	Shoot	118	4	104	6
Artemisia	Root	114	3	110	5
Fennel	Seed	108	2	89	6

Table 6 Recovery data jasmonic acid (JA) in plant matrices at low and high spike concentrations

Species	Tissue	Low recovery average (%)	Low recovery %RSD	High recovery average (%)	High recovery %RSD
SJW	Shoot	87	2	89	2
SJW	Root	86	2	87	3
African violet	Shoot	85	2	86	3
Banana	Shoot	85	2	87	3
Banana	Root	92	8	86	3
Elm	Shoot	85	3	85	3
Tobacco	Shoot	84	2	83	3
Tobacco	Root	83	2	83	3
Potato	Shoot	82	3	82	3
Artemisia	Shoot	85	4	83	4
Artemisia	Root	81	2	80	3
Fennel	Seed	90	1	93	2

Table 7 Recovery data abscisic acid (ABA) in plant matrices at low and high spike concentrations

Species	Tissue	Low recovery average (%)	Low recovery %RSD	High recovery average (%)	High recovery %RSD
SJW	Shoot	93	5	100	4
SJW	Root	84	4	97	3
African violet	Shoot	87	4	97	2
Banana	Shoot	87	4	99	3
Banana	Root	89	4	98	3
Elm	Shoot	88	4	99	3
Tobacco	Shoot	90	4	99	3
Tobacco	Root	91	3	100	3
Potato	Shoot	93	3	101	2
Artemisia	Shoot	96	3	104	2
Artemisia	Root	95	3	104	2
Fennel	Seed	104	3	105	1

Table 8 Recovery data gibberellic acid (GA3) in plant matrices at low and high spike concentrations

Species	Tissue	Low recovery average (%)	Low recovery %RSD	High recovery average (%)	High recovery %RSD
SJW	Shoot	92	2	102	2
SJW	Root	88	2	101	1
African violet	Shoot	92	4	100	2
Banana	Shoot	86	2	102	2
Banana	Root	98	10	102	1
Elm	Shoot	88	2	100	1
Tobacco	Shoot	88	2	99	1
Tobacco	Root	89	3	100	1
Potato	Shoot	92	4	101	1
Artemisia	Shoot	92	5	103	1
Artemisia	Root	103	10	111	8
Fennel	Seed	98	3	103	2

Table 9 Recovery data zeatin in plant matrices at low and high spike concentrations

Species	Tissue	Low recovery average (%)	Low recovery %RSD	High recovery average (%)	High recovery %RSD
SJW	Shoot	90	6	96	2
SJW	Root	85	5	93	2
African violet	Shoot	90	6	99	2
Banana	Shoot	89	6	97	1
Banana	Root	93	6	99	1
Elm	Shoot	92	5	102	1
Tobacco	Shoot	93	5	101	1
Tobacco	Root	96	6	102	1
Potato	Shoot	97	6	106	3
Artemisia	Shoot	97	5	104	2
Artemisia	Root	99	5	107	1
Fennel	Seed	101	1	105	5

Table 10 Recovery data benzylaminopurine (BA) in plant matrices at low and high spike concentrations

Species	Tissue	Low recovery average	Low recovery %RSD	High recovery average	High recovery %RSD
SJW	Shoot	86	3	93	3
SJW	Root	97	3	96	0
African violet	Shoot	93	3	95	1
Banana	Shoot	96	3	97	1
Banana	Root	98	3	96	1
Elm	Shoot	100	3	99	1
Tobacco	Shoot	101	3	102	1
Tobacco	Root	107	3	104	1
Potato	Shoot	104	2	104	2
Artemisia	Shoot	114	5	105	2
Artemisia	Root	107	2	105	1
Fennel	Seed	98	3	106	2

jasmonate allowed for inclusion of these important plant signaling molecules (Table 12) (Figs. 3, 4).

Discussion

Phytohormones, plant hormones, or PGRs are defined by their roles as signaling molecules and by their activity at very low concentrations. This aspect of low biological concentrations makes them innately more difficult to quantify in tissues as compared to other phytochemicals such as pigments, carbohydrates or phenylpropanoids which play structural and functional roles in plants and are present in very large quantities. Due to their essential and significant effects in plants, however, the presence of PGRs in tissues and the quantities at which they are present can be a source of information in deciphering not only the likely growth outcome of a plant, but also the mechanisms underlying morphogenetic and developmental responses (Werner and Schmülling 2009; Enders and Strader 2015).

In vitro culture or plant tissue culture depends on the exogenous application of PGRs to achieve desired diverse morphogenetic outcomes and has applications which span from protoplast isolation and development, to large scale bioreactor production of thousands, even millions of plants (Arteca 1996; Gaspar et al. 1996; Murch et al. 2004; Jones et al. 2014). An understanding of the underlying mechanisms which mediate these processes can not only allow researchers much insight into how these processes occur in plant processes but also allow for the faster development of new protocols and improvement of existing protocols, through an understanding of the fundamental biological interactions which govern these processes. In particular, the ability to determine endogenous concentrations of PGRs in recalcitrant species provides a valuable tool in the culture protocol development pipeline, and may allow for enhanced efficiency of protocol development by streamlining the number of factors which are tested e.g. incorporation of PGR inhibitors or PGR supplementation levels.

Though the importance of the presence and quantities of PGRs in plant tissues and cultures has been recognized, the ability to conduct these experiments is often out of reach for plant science labs. This may be due to various factors such as lack of funding for expensive and specialized equipment, a lack of expertise or experience or requirements for complex or hazardous chemical extraction procedures. A significant consideration in designing any experiment, therefore, is access to equipment, simplicity of analysis and user expertise. The method presented in this paper is simple and straightforward to complete and

Table 11 Recovery data 6-(γ,γ-dimethylallylamino)purine (2-iP) in plant matrices at low and high spike concentrations

Species	Tissue	Low recovery average (%)	Low recovery %RSD	High recovery average (%)	High recovery %RSD
SJW	Shoot	98	1	102	1
SJW	Root	103	1	105	1
African violet	Shoot	101	1	105	1
Banana	Shoot	91	3	94	2
Banana	Root	108	1	109	0
Elm	Shoot	105	2	108	1
Tobacco	Shoot	108	2	110	0
Tobacco	Root	111	2	112	0
Potato	Shoot	111	2	110	1
Artemisia	Shoot	112	2	116	4
Artemisia	Root	110	2	112	1
Fennel	Seed	101	1	99	2

Table 12 Summary of changes to the methods required for addition of phytohormone derivatives

Analyte	Retention time (min)	m/z	Ionization mode	Cone voltage (V)	Changes to LC method?
ASA	0.93	137	ESI+	10	None
OPDA	4.05	291	ESI−	15	Extend gradient to 5 min
JA-Ile	3.88	322	ESI−	15	Extend gradient to 5 min

ASA acetyl salicylic acid, *OPDA* 12-oxophytodienoic acid, *JA-Ile* jasmonic acid isoleucine

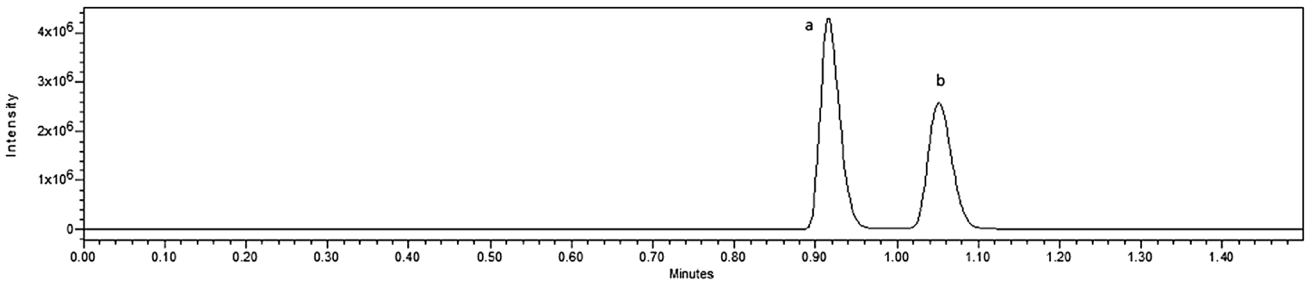

Fig. 3 Chromatogram of acetyl salicylic acid (*a*) added to salicylic acid (*b*) channel (created in Empower 3, Waters)

employs a user friendly analytical system which does not require in-depth analytical chemistry experience on the part of the user, or use of complex extraction procedures. Unlike other published protocols which may require specialized extraction equipment such as is required for solid phase extraction (SPE) or difficult to handle or hazardous chemicals such as ethers or ethyl acetate, this method requires a single extraction step with a relatively inexpensive and easily disposed extraction solvent and does not require further solvent removal or sample concentration prior to analysis. Additionally, due to the relatively low cost of the system this method may be more accessible than comparable methods on tandem and time of flights mass spectrometry systems, while still providing a large increase in sensitivity and selectivity (Bu et al. 2016) (Table 2; Fig. 2).

Due to the chemical diversity of plant species, and even of tissues within a given species, this report utilized twelve different plant matrices. These matrices were chosen to represent species which spanned agricultural, medicinal and model species and investigated three different tissue types: roots, shoots (considered to be leaves and stems) and seed, which represent different chemical environments within a plant. A total of eight species from seven plant families are represented. This method was found to be highly reproducible across all PGRs and tissues tested. The cytokinins showed the lowest variability with %RSD as low as 0% in the high concentration spikes and 1% in low concentration

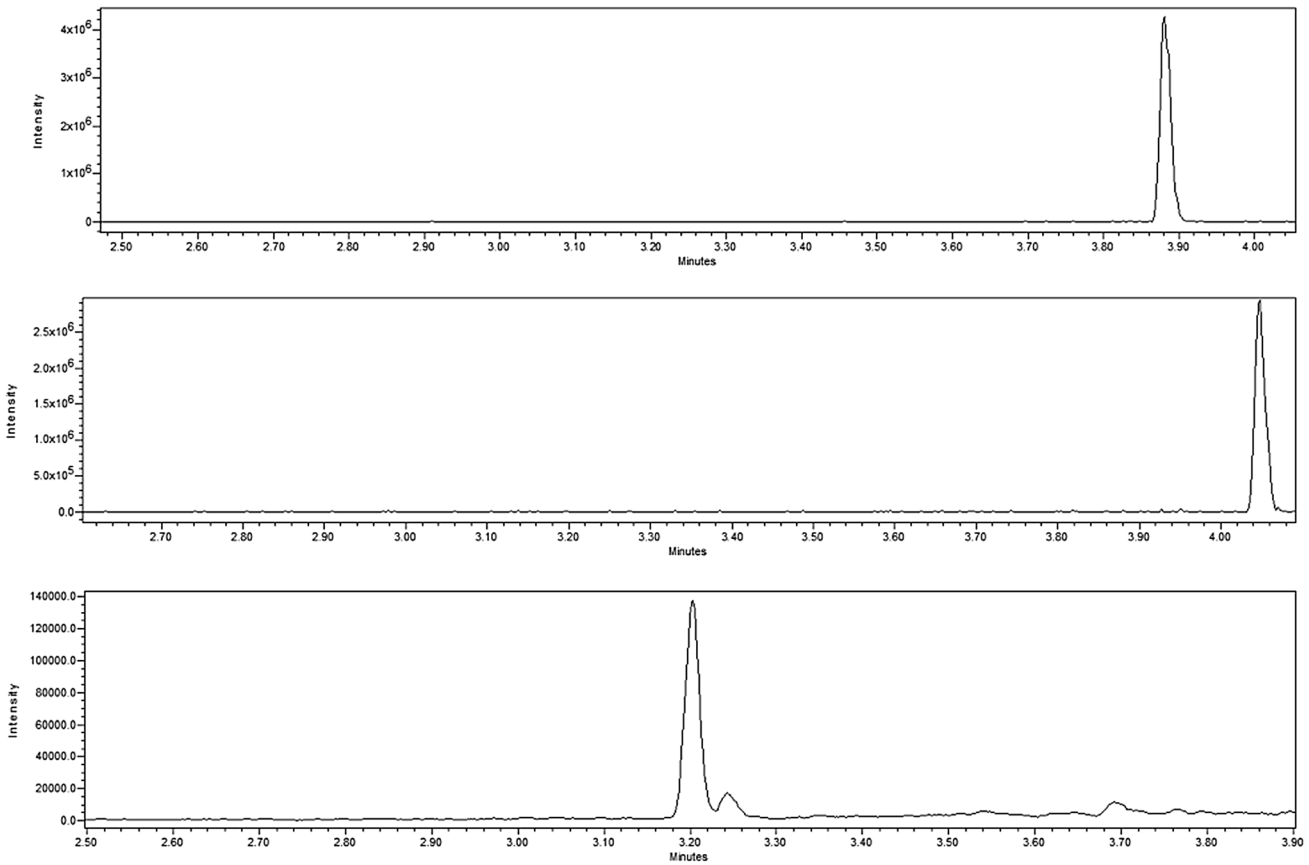

Fig. 4 Chromatograms for individual jasmonate. *Top* jasmonic acid isoleucine, *middle* 12-oxo-phytodienoic acid, *bottom* jasmonic acid (created in Empower 3, Waters)

spikes, however, all analytes and matrices showed very good reproducibility with variability of no more than 10% in any given sample matrix. This demonstrates that this method can be utilized across a diversity of plant samples for repeated experiments and achieve consistent results.

Recovery data demonstrated the accuracy of the method with all recoveries being well within 20% of the known concentration spiked into the matrix for both low and high concentrations. Matrices did not appear to have a significant effect on any analyte in this method, therefore this method is accurate for the quantification of PGRs in a wide diversity of species and tissue types.

Though this method is validated for the quantification of several primary plant growth regulators in plants tissues, oftentimes the primary form which may be used for treatment in tissue culture is not the biologically active form, or there may exist other synthetic derivatives of a PGR. In this case it may be desirable to also investigate the presence and quantity of these compounds in the sample. Two such examples, which were investigated in this study, are the jasmonates OPDA and JA-Ile and the salicylate ASA (Fig. 1). Though JA is often used in the treatment of plants, it is not the bioactive form in plant tissues. *In planta*, the JA conjugate JA-Ile, is the bioactive form which is involved in many plant processes, while OPDA is the main biosynthetic precursor for JA, with these compounds playing important roles with regards to plant defense and immunity (Sherif et al. 2016). ASA is a synthetic derivative of SA, which has been used in numerous plant tissue culture experiments and has in some cases been found to have enhanced or differential activity as compared to its endogenous relative, possibly due to differences in bioavailability or differential induction of signaling cascades (Hutchinson and Saxena 1996). It is, therefore, often desirable to identify and quantify these compounds or other PGR derivatives in plant tissues to allow for investigation of these processes. Through inclusion of ASA with no modification to the method parameters and inclusion of JA-Ile and OPDA with only minor modifications, this method has proven to be an excellent starting point for development of methods for the inclusion of other PGRs. This could be particularly applicable for other classes of PGRs with diverse chemicals structures, such as auxins e.g. indole-3-butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D) or gibberellins e.g. gibberellic acid 1 (GA1) or

gibberellic acid 4 (GA4), for example. A number of compounds are currently used in in vitro propagation of plant species mechanism of whose action involves modulation of endogenous auxin, cytokinin or ethylene complement. The efficacy of such compounds can be further increased with knowledge of accurate levels of PGRs in cultured cells tissues.

Conclusion

This report presents a rapid method for the analysis for the major classes of plant growth regulators which are utilized in plant biology and plant tissue culture systems. It requires a relatively simple, low-risk and low cost platform for analysis and does not require extensive analytical chemistry knowledge on the part of the operator. Additionally, it has been demonstrated, through the addition of three PGR derivatives, to be an excellent starting point for the development of further methods. This, therefore, will provide a new tool in overcoming recalcitrance in plant tissue culture and help to enable plant science researchers to further understand the phytochemical landscape, particularly with respect to PGRs and help to increase understanding and efficiency of plant morphogenetic systems.

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Author contributions LAEE participated in conception and design, data acquisition, analysis and interpretation, MRS participated in conception and design, WBG participated in conception and design and data analysis and PKS participated in conception and design and data interpretation. All authors participated in manuscript preparation and gave final approval of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

AOAC International (2013) Appendix K: Guidelines for dietary supplements and botanicals. In: Official methods of analysis. AOAC International, Arlington, Appendix K: 1–32

Arteca RN (1996) Manipulation of growth and photosynthetic processes by plant growth regulators. In: Plant growth substances. Springer, Boston, pp 240–272

Assani A, Chabane D, Foroughi-Wehr B, Wenzel G (2006) An improved protocol for microcallus production and whole plant regeneration from recalcitrant banana protoplasts (*Musa* spp.). Plant Cell Tissue Organ Cult 85:257–264. doi: 10.1007/s11240-005-9058-y

Betz JM, Brown PN, Roman MC (2011) Accuracy, precision, and reliability of chemical measurements in natural products research. Fitoterapia 82:44–52. doi: 10.1016/j.fitote.2010.09.011

Bliesner DM (2005) Validating chromatographic methods: a practical guide. Wiley, Hoboken

Bonga JM, Klimaszewska KK, von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tissue Organ Cult 100:241–254. doi: 10.1007/s11250-009-9647-2

Bu X, Regalado EL, Hamilton SE, Welch CJ (2016) The emergence of low-cost compact mass spectrometry detectors for chromatographic analysis. Trends Anal Chem 82:22–34. doi:10.1016/j.trac.2016.04.025

Cai B-D, Ye E-C, Yuan B-F, Feng Y-Q (2015) Sequential solvent induced phase transition extraction for profiling of endogenous phytohormones in plants by liquid chromatography-mass spectrometry. J Chromatogr B 1004:23–29. doi: 10.1016/j.jchromb.2015.09.031

Cutler AJ, Saleem M, Coffey MA, Loewen MK (1989) Role of oxidative stress in cereal protoplast recalcitrance. Plant Cell Tiss Org Cult 18:113–127. doi:10.1007/BF00033470

Enders TA, Strader LC (2015) Auxin activity: past, present, and future. Am J Bot 102:180–196. doi: 10.3732/ajb.1400285

Gantait S, Sinniah UR, Ali MN, Sahu NC (2015) Gibberellins—a multifaceted hormone in plant growth regulatory network. Curr Protein Pept Sci 16:406–412. doi: 10.2174/1389203716666150440125439

Gaspar T, Kevers C, Penel C, Greppin H (1996) Plant hormones and plant growth regulators in plant tissue culture. In Vitro Cell Dev Biol Plant 32:272–289

Hewezi T, Jardinaud F, Alibert G, Kallerhoff J (2003) A new approach for efficient regeneration of a recalcitrant genotype of sunflower (*Helianthus annuus*) by organogenesis induction on split embryonic axes. Plant Cell Tissue Organ Cult 73:81–86. doi:10.1023/A:1022689229547

Holopainen JK, Blande JD (2012) Molecular plant volatile communication. Adv Exp Med Biol 739:17–31. doi: 10.1007/978-1-4614-1704-0_2

Huang G-T, Ma S-L, Bai L-P et al (2012) Signal transduction during cold, salt, and drought stresses in plants. Mol Biol Rep 39:969–987. doi: 10.1007/s11033-011-0823-1

Hutchinson MJ, Saxena PK (1996) Acetylsalicylic acid enhances and synchronizes thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Bailey) tissue cultures. Plant Cell Rep 15:512–515. doi: 10.1007/BF00232984

Jones AMP, Shukla MR, Biswas GCG, Saxena PK (2014) Protoplast-to-plant regeneration of American elm (*Ulmus americana*). Protoplasma 252:925–931. doi: 10.1007/s00709-014-0724-y

Kaur P, Kothari SL (2004) In vitro culture of kodo millet: anfluence of 2,4-D and picloram in combination with kinetin on callus initiation and regeneration. Plant Cell Tissue Organ Cult 77:73–79. doi: 10.1023/B:TICU.0000016505.20448.44

Li H, Murch SJ, Saxena PK (2000) Thidiazuron-induced de novo shoot organogenesis on seedlings, etiolated hypocotyls and stem segments of Huang-qin. Plant Cell Tissue Organ Cult 62:169–173. doi: 10.1023/A:1006491408762

Li G, Lu S, Wu H et al (2015) Determination of multiple phytohormones in fruits by high-performance liquid chromatography with fluorescence detection using dispersive liquid-liquid microextraction followed by precolumn fluorescent labeling. J Sep Sci 38:187–196. doi: 10.1002/jssc.201401131

Mithila J, Hall J, Victor JMR, Saxena P (2003) Thidiazuron induces shoot organogenesis at low concentrations and somatic embryogenesis at high concentrations on leaf and petiole explants of African violet (*Saintpaulia ionantha* Wendl.). Plant Cell Rep 21:408–414. doi: 10.1007/s00299-002-0544-y

Moubayidin L, Di Mambro R, Sabatini S (2009) Cytokinin–auxin crosstalk. Trends Plant Sci 14:1360–1385. doi: 10.1016/j.tplants.2009.06.010

Mundhara R, Rashid A (2006) Recalcitrant grain legume *Vigna radiata*, mung bean made to regenerate on change of hormonal and cultural conditions. Plant Cell Tissue Organ Cult 85:265–270. doi: 10.1007/s11240-005-9061-3

Murch SJ, Saxena PK (2001) Molecular fate of thidiazuron and its effects on auxin transport in hypocotyls tissues of *Pelargonium* × *hortorum* Bailey. Plant Growth Regul 35:269–275. doi:10.1023/A:10144689059593

Murch SJ, Liu C, Romero RM, Saxena PK (2004) In vitro culture and temporary immersion bioreactor production of *Crescentia cujete*. Plant Cell Tissue Organ Cult 78:63–68. doi:10.1023/B:TICU.0000020397.01895.3e

Murthy B, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of in vitro plant morphogenesis. In Vitro Cell Dev Biol Plant 34:267–275. doi 10.1007/BF02822732

Murthy HN, Hahn EJ, Paek KY (2008) Recurrent somatic embryogenesis and plant regeneration in *Coriandrum sativum* L. Sci Hortic 118:168–171. doi: 10.1016/j.scienta.2008.05.037

Nguyen AH, Hodgson LM, Erskine W, Barker SJ (2016) An approach to overcoming regeneration recalcitrance in genetic transformation of lupins and other legumes. Plant Cell Tissue Organ Cult 127:623–635. doi: 10.1007/s11240-016-1087-1

Pan X, Welti R, Wang X (2010) Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. Nat Protoc 5:986–992. doi: 10.1038/nprot.2010.37

Pliego-Alfaro F, Monsalud MJR, Litz RE, Gray DJ, Moon PA (1996) Effect of abscisic acid, osmolarity and partial desiccation on the development of recalcitrant mango somatic embryos. Plant Cell Tissue Organ Cult 44:63–70. doi: 10.1007/BF00045914

Sanago MH, Murch SJ, Slimmon TY, Krishnaraj S, Saxena PK (1995) Morphoregulatory role of thidiazuron: morphogenesis of root outgrowths in thidiazuron-treated geranium (*Pelargonium* × *hortorum* Bailey). Plant Cell Rep 15:205–211. doi: 10.1007/BF00193721

Sherif S, Jones A, Shukla MR, Saxena PK (2014) Establishment of invasive and non-invasive reporter systems to investigate American elm–*Ophiostoma novo-ulmi* interactions. Fungal Genet Biol 71:32–41. doi: 10.1016/j.fgb.2014.08.005

Sherif SM, Shukla MR, Murch SJ et al (2016) Simultaneous induction of jasmonic acid and disease-responsive genes signifies tolerance of American elm to Dutch elm disease. Sci Rep 6:21934. doi: 10.1038/srep21934

Shukla MR, Jones AMP, Sullivan JA et al (2012) *In vitro* conservation of American elm (*Ulmus americana*): potential role of auxin metabolism in sustained plant proliferation. Can J For Res 42:686–697. doi: 10.1139/x2012-022

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol 11:118–130

Song S, Qi T, Wasternack C, Xie D (2014) Jasmonate signaling and crosstalk with gibberellin and ethylene. Curr Opin Plant Biol 21:112–119. doi: 10.1016/j.pbi.2014.07.005

Werner T, Schmülling T (2009) Cytokinin action in plant development. Curr Opin Plant Biol 12:527–538. doi: 10.1016/j.pbi.2009.07.002

Xu X, Xie G, He L, Zhang J, Xu X, Qian R, Liang G, L J-H (2013) Differences in oxidative stress, antioxidant systems, and microscopic analysis between regenerating callus-derived protoplasts and recalcitrant leaf mesophyll-derived protoplasts of *Citrus reticulata* Blanco. Plant Cell Tissue Organ Cult 114:161–169. doi: 10.1007/s11240-013-0312-4