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Review

The Use of Auxin Quantification for Understanding Clonal Tree Propagation

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Abstract: Qualitative and quantitative hormone analyses have been essential for understanding the metabolic, physiological, and morphological processes that are influenced by plant hormones. Auxins are key hormones in the control of many aspects of plant growth and development and their endogenous levels are considered critical in the process of adventitious root induction. Exogenous auxins are used extensively in the clonal propagation of tree species by cuttings or tissue culture. Understanding of auxin effects has advanced with the development of increasingly accurate methods for auxin quantification. However, auxin analysis has been challenging because auxins typically occur at low concentrations, while compounds that interfere with their detection often occur at high concentrations, in plant tissues. Interference from other compounds has been addressed by extensive purification of plant extracts prior to auxin analysis, although this means that quantification methods have been limited by their expense. This review explores the extraction, purification, and quantification of auxins and the application of these techniques in developing improved methods for the clonal propagation of forestry trees.

Keywords: adventitious rooting; auxin; cloning; cuttings; indole-3-acetic acid; indole-3-butyric acid; indolyl-3-acetic acid

1. Introduction

Plant hormones regulate all stages of growth and development from embryogenesis to senescence [1,2]. Plant hormones include auxins, gibberellins, cytokinins, abscisic acid, ethylene, jasmonates, brassinosteroids, salicylates, and strigolactones [3,4]. Knowledge of the structure and activity of the first identified group of plant hormones, the auxins, arose in the early-20th century from isolation of one of the predominant endogenous auxins, indole-3-acetic acid (IAA), and measurement of the curvature and elongation of seedling shoots and roots in simple biological experiments [5–9]. Analysis methods evolved during the mid-20th century to include more-complex bioassays that allowed quasi-quantification of auxin levels [9–11]. More-accurate and more-precise information on plant hormone concentrations was long considered a requirement for clearly establishing the connections between auxins and their physiological effects [12,13]. This requirement was met with the development of instrumental techniques that provided definitive auxin identification and accurate quantification. These methods helped to demonstrate the active forms of auxin that are involved in plant development, and to show that auxins often act through their effects on cell division or the orientation of cell expansion [10,14–18].

The concentrations of endogenous auxins in plants depend on their rates of biosynthesis, transport, conjugation, and catabolism [19]. Indole-3-acetic acid (IAA) is one of the main endogenous auxins, but it is relatively labile [20]. Other endogenous auxins, such as indole-3-butyric acid (IBA), are more stable and travel more efficiently by polar transport [20,21]. Polar transport down the stem is the main mechanism of transport of auxins that are synthesized in apical meristems [2,13,14]. Auxins are now considered important regulators of, for example, embryo development, meristem organization, apical dominance, and lateral root formation [1,2,14,16,22].

Auxins are also critical regulators of adventitious root induction (i.e., the formation of roots from non-root tissues) [23,24]. Adventitious root formation consists of three phases: root induction, root initiation, and root emergence [25,26]. The induction phase is the period during which biochemical changes occur prior to the first cell division; initiation refers to the cell divisions that lead to the formation of root primordia; emergence refers to the growth of adventitious roots through the cortex and out of the epidermis. Endogenous IAA levels often increase temporarily during the induction phase while, simultaneously, the plant tissue possesses increased sensitivity to auxin signals [27]. Auxin levels decline during the initiation phase [26,28]. Auxins are directly involved in root induction [29], but they can exert inhibitory effects during the initiation and emergence phases [30,31]. However, the endogenous production of auxins in apical meristems is not always adequate to induce adventitious roots in stem tissues [32].

Exogenous auxins, in particular indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA), are commonly used to induce adventitious rooting during clonal propagation from cuttings or tissue cultured shoots [33–36]. Auxin application often increases the percentage of shoots that forms roots and the number of adventitious roots that are formed in each shoot [36–39]. However, many woody plant species are difficult to propagate from cuttings or tissue culture, especially when the shoots have been sourced from mature trees [40–43]. The ability to isolate and accurately quantify auxins and their metabolites is likely to be fundamental in identifying markers for tree maturation and developing improved methods for clonal propagation [41,43–45].

Auxin quantification has traditionally been difficult because of the trace levels of auxins in plant tissues and the high concentrations of compounds that interfere with auxin detection and quantification [3,46,47]. Extensive sample purification [48,49], the addition of isotopically-labelled hormone molecules as internal standards [11,50,51], high-performance instrumental techniques [9], and new developments in laboratory automation [52,53] have all raised the capacity to isolate and quantify auxins. This paper reviews techniques for identifying and quantifying auxins with an emphasis on forest tree species, and it compares the advantages, limitations, and applications of these techniques for improving forestry tree production.

2. Techniques for Auxin Quantification

2.1. Bioassays

The first endogenous auxin quantification was performed by simple bioassays in the early 20th century [5,12,54]. Bioassays were generally based on the diffusion of sample auxin, captured in agar blocks, into the coleoptiles of *Avena sativa* L. seedlings. The degree of ensuing coleoptile curvature was related to the auxin concentration in the original sample. One extraction method, described as a simple diffusion method, was based on auxin diffusion from the plant tissue into the agar blocks. This method was most suited to determining auxin production and movement from small samples such as apical meristems [55,56]. The method allowed auxin to diffuse through overlapping agar blocks, and each block was then applied to an *A. sativa* coleoptile to measure its curvature. The auxin content of the original sample could be calculated based on theoretically- and experimentally-derived diffusion tables that accounted for the diffusion duration and temperature [57,58]. Another extraction method used solvents to extract auxin from the plant tissue, followed by quantification using *A. sativa* coleoptiles. Quantification was improved greatly by gradually optimizing the extraction solvents. The first auxin

extraction method used chloroform and hydrochloric acid as solvents [54]. Subsequent studies used alcohol [59], ether and acetic acid [6], or ether and hydrochloric acid [60]. Ether extraction was more effective than extraction with chloroform, ethanol, and ethanol in sequence, although the process took several months [61]. Extraction using ethanol, chloroform, and water subsequently allowed estimates of auxin content with just a 15-min extraction period [62]. Auxin could also be extracted rapidly by freezing intact tissue in dry ice and using ether as a solvent in two to three stages of 30 min each at room temperature [63]. Four consecutive 30-min extractions using ether were also effective for auxin extraction at room temperature [64]. However, bioassay methods gradually became obsolete because of their low specificity for auxin, imprecise results, and the long durations required for bioassay plant preparation [65–69].

2.2. Immunoassays

The advent of immunocytochemistry led to immunoassay techniques for quantifying auxins and other plant hormones [70,71]. Immunoassays are based on the production of proteins (antibodies) that recognize and connect to specific compounds (i.e., antigens) [72,73]. Antibodies are raised, for example, by conjugating IAA to bovine serum albumin, injecting the conjugated IAA into rabbits, collecting a blood sample from the animals, and testing the antisera for their affinity and specificity to auxin compounds [74,75]. The effectiveness of immunoassays for plant hormone quantification was improved greatly with radioimmunoassay (RIA) and enzyme immunoassay (EIA) techniques [76,77]. Radioimmunoassay (RIA) is a sensitive method used to measure the concentrations of compounds based on competition at antibody binding sites between an antigen (e.g., sample IAA) and a radiolabeled analog (e.g., tritiated IAA) [78]. Enzyme immunoassay (EIA) consists of a series of reactions between antibodies and antigens that result in binding of an enzyme-labeled antibody to a solid phase [79]. EIA typically requires less expensive equipment and may provide greater sensitivity in detecting plant hormones than RIA [80]. The requirements of RIA for radioactively-labelled hormones and a scintillation counter also make this technique impractical in some laboratories [80].

Enzyme-linked immunosorbent assay (ELISA) improved the reliability of endogenous auxin quantification [77,81]. ELISA methods include indirect competitive ELISA (icELISA) and direct competitive ELISA (dcELISA) methods [82]. The icELISA is an immobilized antigen assay, based on competition between an immobilized antigen and an unknown amount of analyte for a small fixed amount of antibody, with the activity of immobilized antigen bound to the antibody being determined using a second antibody labeled with the enzyme. The dcELISA is an immobilized antibody specific assay, based on competition between a constant amount of hapten-enzyme and the analyte for a fixed amount of antibody [82,83]. ELISA methods have enabled precise quantification of free and conjugated endogenous auxin levels during seed development of Douglas fir [84]. A solid-phase enzyme immunoassay using specific anti-hormone antibodies has been used for the quantification of IAA, abscisic acid (ABA), and zeatin plus zeatin riboside (Z + ZR) in cuttings of *Lycopersicon esculentum* Mill. during the first five days of adventitious rooting [85]. Free and conjugated IAA in *Picea abies* (L.) H.Karst. and *Pinus radiata* D.Don needles have also been quantified using ELISA in the solid phase [44,86,87]. The availability of commercial kits contributed to the popularity of ELISA for the quantification of plant hormones [77,78,81,88]. These kits allowed the analysis of multiple samples in microtiter plates [89–92]. Immunoenzymatic techniques are considered to have high specificity for plant hormones, but the long periods required for antibody preparation [69], the cross reactivity of antibodies with multiple compounds from the same hormone class [93–95], and the possibility of interference from other compounds [88,96] have been major limitations.

2.3. Chromatography and Mass Spectrometry

Physicochemical techniques, such as high performance liquid chromatography (HPLC) and gas chromatography—mass spectrometry (GC-MS), have enabled the quantification of hormones and their metabolites with much greater precision, sensitivity, and speed [1,9,97,98]. Chromatographic methods

for auxin quantification date from the mid-20th century [99–102] including early use on forestry trees such as *Salix* × *fragilis* L. [103], *Picea abies* [104], and *Populus tremula* L. [105]. Chromatographic methods can provide excellent hormone separation and, when coupled with mass spectrometry (MS), allow quantification of compounds from multiple hormone classes in the same sample [69,106]. These methods have greatly improved endogenous auxin identification in forestry species [107–110].

HPLC has been one of the most widely used separation techniques in auxin analysis [111]. HPLC has been used for extract purification both in herbaceous species [112] and forest species (e.g., *Populus tremula* × *P. tremuloides* Michx.) [113]. HPLC with diode-array ultra-violet (UV) detection was used to quantify seasonal variation in IAA and ABA concentrations in *Eucommia ulmoides* Oliv. trees, demonstrating an abrupt reduction in IAA concentrations and an increase in ABA concentrations in cold periods, and confirming an interaction between IAA and ABA in cambial dormancy [114]. However, UV or fluorescence detectors may provide inaccurate results because other compounds in the same HPLC fractions may have similar UV absorbance or fluorescence spectra [9]. HPLC-MS has been used to quantify IAA in *Pinus radiata* needles and determine auxin effects on the plasticity of resistance to water stress in different climatic regions [115]. Provenances that were less tolerant to water stress displayed an increase of more than 10× the concentrations of IAA by the end of the dry period, when compared with control provenances.

HPLC has been considered more efficient since the introduction of second dimension HPLC (2D-HPLC), in which all the molecules of all sample components (or a large, constant aliquot of them) migrate along the columns used in the two dimensions of the separation and are eluted from the second column [116]. This is an effective purification technique when the number of substances to be purified is limited. Features contributing to the popularity of 2D-HPLC are its high purification potential, reproducibility, robustness, high throughput, and unattended operation [96]. Another method for effective quantitative analysis of auxins is liquid chromatography coupled to tandem mass spectrometry with electrospray ionization (HPLC-ESI-MS/MS) [117]. This technique has been used to simultaneously determine the levels of different plant hormones, including IAA. The addition of labelled deuterium before extraction improves the technique precision, significantly reducing the time required for sample preparation and hormone analysis [118]. Capillary electrophoresis (CE) is another technique that has been used for auxin purification. CE allows rapid and efficient separation of charged components present in small sample volumes. The separations are based on the differences in mobility of ions in electrophoretic media inside small capillaries [119,120]. Capillary electrophoresis-electrochemiluminescence (CE-ECL) has been applied for IAA and ABA quantification in *Acacia* sp. young leaves and buds and in *Vigna radiata* (L.) R.Wilczek sprouts [121]. CE-ECL is considered a promising technique because it provides high resolving power, sensitivity, and low sample consumption. However, the requirement for high extract purity has been a major limitation of the CE technique [96].

Mass spectrometry was originally adapted for IAA quantification from samples of herbaceous species [112] and GC-MS was used increasingly for IAA purification and quantification. The advent of capillary columns in the 1980s greatly improved both the resolving power and the sensitivity of GC-MS [9]. The use of GC-MS for auxin analysis in forest species commenced with research on *Pseudotsuga menziesii* (Mirb.) Franco [122–124]. GC-MS was later applied to assess seasonal variation in IAA concentrations in *Pinus sylvestris* Thunb. seedlings [125] and to investigate the influence of IAA concentration and distribution on xylem development in transgenic *Populus tremula* × *P. tremuloides* [113]. GC-MS has also been used to quantify free IAA levels in vascular tissues of *Populus* × *tomentosa* Carrière [126], to show that the highest IAA oxidase activity in *Ginkgo biloba* L. leaves occurs in environments with high CO_2 and C concentrations [127], and to demonstrate that IAA contents are correlated with IAA oxidase activity in *Pinus tabuliformis* Carrière needles exposed previously to high CO_2 concentrations [128]. Recently, GC-MS has been used to measure IAA concentrations and identify their relationships with rooting capacity in *Corymbia torelliana* (F.Muell.) K.D.Hill & L.A.S.Johnson × *C. citriodora* (Hook.) K.D.Hill & L.A.S.Johnson cuttings [110,129].

Mass spectrometry requires a high degree of sample purification and derivatization [96]. Derivatization, with the objective of increasing volatility and sensitivity to distinguish methyl esters from free acids, is a critical step in obtaining adequate purification by GC [130]. Whole-plant organ-distribution maps for indole-3-acetic acid, abscisic acid, jasmonic acid, 12-oxo-phytodienoic acid, and salicylic acid have been generated for *Arabidopsis thaliana* (L.) Heynh. using GC-MS/MS preceded by sample purification in one or two solid-phase-extraction (SPE) columns [131]. Liquid chromatography coupled with mass spectrometry (LC-MS) [117,132–135] has become much more widely used because of its high capacity to separate and detect plant hormones without derivatization but with high sensitivity and efficiency [71]. Ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) is now one of the most common approaches for quantification of auxins and their conjugates [136–140], often employing triple quadrupole mass spectrometers that improve specificity and reduce detection limits [136–139]. Recent improvements in GC-MS/MS and LC-MS/MS techniques for auxin analysis have been the focus of comprehensive reviews [9,135].

Conjugation of IAA may be a mechanism used by plants to, in effect, regulate IAA levels by storing IAA and protecting it against degradation [14]. The conjugation of free IAA with some amino acids results in more-hydrolysable, biologically-inactive compounds, such as IAA-alanine (IAA-Ala) and IAA-aspartate (IAA-Asp) [13]. Shifts in the equilibrium between IAA conjugation and deconjugation can affect rooting of plant tissues [141]. Quantification of conjugated IAA involves a more elaborate process than quantification of free IAA, especially because of the significantly lower concentrations of the conjugates often present in tissues [73]. Prior enrichment of auxin conjugates has been used during extract purification in association with various methods of quantification, such as immunoaffinity for *Helleborus niger* L. [73] or coupled with spectrometry of single-quad mass in seedlings of *Pinus radiata* [142]. Solid phase extraction (SPE) followed by methylation and separation by HPLC has been a simple and efficient protocol for purifying IAA and some of its conjugates in *Arabidopsis thaliana* [143]. SPE prior to HPLC-MS/MS analysis, without previous derivatization, allowed quantification of IAA conjugated with Ala, Asp, and Glu in samples of *Oryza sativa* L. [117].

Two promising techniques for identifying the cellular and subcellular locations of endogenous auxins are matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MSI) and secondary ion mass spectrometric imaging (SIMS-MSI). These techniques provide simultaneous high-resolution identification and imaging of compounds to visualize their distribution in plant tissue [144–147]. They have been applied extensively to study the distribution of drugs and their metabolites in human and animal tissues [145,148]. MALDI-MS has been used recently to visualize the tissue-specific locations of 48 metabolites during germination of *Hordeum vulgare* L. seed [149] and to identify taxanes from *Taxus canadensis* Marshall needles [150] and monolignol glucosides in *Chamaecyparis obtusa* (Siebold and Zucc.) Endl. and *Cryptomeria japonica* (Thunb. Ex L.f.) D.Don wood [151]. These approaches have great potential for visualizing the distribution of endogenous and exogenous auxins, and their conjugates, in plant tissues.

3. Auxin Dynamics during Vegetative Propagation

Quantitative analysis of endogenous auxin levels has contributed greatly to understanding and improving vegetative propagation [45]. The production of synthetic auxins in the 20th century also led to research into their effects on adventitious root induction [152,153]. Much of this research has focused on determining the appropriate combinations of auxin concentration, application formulation, and application method that result in the highest percentages of shoots forming roots [109,154,155]. Auxins are used primarily to increase plant production by improving rooting percentages, but they can also improve plant quality parameters such as adventitious root number, root system symmetry, and root:shoot ratio [37,38,155,156]. These parameters can have important impacts on tree stability, tree survival, and trunk volume in the nursery and the plantation. For example, raising the number of

adventitious roots from one to five increases nursery survival by 11% [157], tree height after two years by 23% [158], and trunk diameter after five years by 12% [159] in various *Pinus* species.

Auxin concentrations in stem cuttings depend directly on the speed of their oxidation and conjugation [31,160], and auxin action may be influenced by phenolic compounds that inhibit or promote rooting [24,161]. Free IAA is quickly metabolized by peroxidase [3,26] whereas, in its conjugated forms such as IAA-Asp, auxin is partially protected against oxidation [49]. Conjugation prevents free-IAA accumulation in tissues [28], and conjugated IBA may be an even-more stable source of auxin in plant tissues because of its greater resistance to enzymatic degradation [20,162]. Peroxidase activity in *Phoenix dactylifera* L. cuttings is at a minimum on the sixth day and at a maximum on the twelfth day after treatment with auxin [163]. Changes in IAA oxidase activity follow a similar pattern, while phenolic contents and auxin protector (enzyme inhibitor) levels show a reverse trend. Auxin concentrations often remain stable even when auxin is applied to *P. dactylifera* cuttings. However, there may be significant differences in the rooting capacity of the auxin-treated and untreated cuttings, suggesting the need for adequate amounts of IAA during the initial root induction phase [163].

Auxin concentrations in cuttings are often highest soon after severance of cuttings from the mother plant. Endogenous IAA concentrations increase during the first 24 h and then decrease over the next 24 h during adventitious root formation in *Vigna radiata* hypocotyl cuttings [26]. Application of auxins to the base of cuttings has, therefore, been recommended to mitigate the effects caused by rapid oxidation of endogenous IAA [3]. IAA or IBA application to *Pisum sativum* cuttings increases internal IAA levels within the first day of application [49]. However, IAA levels return to control levels after 48 hours following IAA application, whereas 70% of applied IBA is still present in the cuttings. Endogenous levels of IAA and IAA-Asp are higher in *Castanea sativa* Mill. × *C. crenata* Siebold and Zucc. cuttings treated with IBA than in control cuttings, with the peak IAA concentration at the base of cuttings occurring on the second day after treatment, after which there is a gradual decrease [164]. Similarly, application of IBA to rootstock of 'Gisela 5' cherry increases endogenous IAA levels within the first day after application [27].

Rooting capacity decreases concomitantly with a reduction in free IAA endogenous concentrations at the base of *Forsythia* × *intermedia* Zabel cuttings [165]. IAA levels are the same in easy-to-root and difficult-to-root clones of *Eucalyptus globulus* Labill., but IAA-Asp levels are higher in the difficult-to-root genotype, suggesting that IAA conjugation plays an important role in determining adventitious rooting capacity in this species [45]. IAA levels in the cutting base and apex remained constant and were similar to those in intact seedlings, indicating that root initiation can occur without an increase in the IAA levels in the root regeneration zone [45]. Differences in auxin transport capacity may contribute directly to success in the rhizogenesis process [108], with applied auxin possibly being transported more rapidly in easy-to-root plants [141].

IBA typically has greater capacity than IAA in inducing adventitious roots [20,29,31]. This capacity can be attributed to its greater stability and lower sensitivity to oxidation [49,162,166]. IBA has been the most widely applied auxin for root induction in woody species because of this high stability compared with IAA and its lower toxicity compared with NAA [33,155,167]. Endogenous IAA levels are higher than endogenous IBA levels in most plant species [21]. However, the relative effectiveness of the two auxins in adventitious root induction depends directly on the stability of their conjugates [166].

Greater propagation success is usually obtained with juvenile, compared with mature, plant material [109,168–170], and auxin concentrations have been postulated as potential biochemical markers of juvenility in tree species [41,42,110,129]. However, IAA concentrations tend to be similar at the base of *Prunus* sp. cuttings derived from mature (40-year-old), semi-mature (5-year-old), and juvenile (in vitro) trees, even following IBA application [109]. Despite having similar endogenous IAA concentrations, the rooting of juvenile *Prunus* cuttings tended to be higher than the rooting of semi-mature and mature cuttings, which could suggest different auxin transport mechanisms in cuttings from the different-aged trees [109]. IAA concentrations are also similar between nodes of *Corymbia torelliana* × *C. citriodora* seedlings that differ in rooting capacity, and also between

cuttings from *C. torelliana* × *C. citriodora* stock plants of different heights that differ in rooting capacity [110,129,171]. The rooting response of a tissue can be regulated by many factors other than auxins, including the sensitivity of cells to the auxin signal [165], the concentrations of rooting inhibitors in the base of cuttings [165], the levels of minerals and carbohydrates [172–178], and the degree of lignification or sclerification of the stem [33,110,179,180].

4. Conclusions

Low auxin concentrations in plants and the presence of compounds that interfere with auxin detection make the quantitative analysis of auxins a challenge for analytical chemistry. Chromatographic methods have been used increasingly to exclude interfering compounds, separate different hormones from each other, and increase the precision of auxin quantification. The combination of GC or LC with MS has been a major advance in the analysis of IAA and other auxins, with LC-MS/MS being used increasingly to analyze auxins directly with minimal prior purification. The assessment of auxin and other hormone levels in plant propagules, and their relationships with rooting and other physiological processes, are important research areas for improving the efficiency of vegetative propagation. The high costs of the analytical instruments required for hormone quantification have been an obstacle limiting their widespread use in forestry research. However, increasingly cost-effective methods to quantify multiple plant hormones in plant samples are likely to greatly improve our understanding of the roles of endogenous and exogenous auxins, and their conjugates, in the process of adventitious root induction.

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