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ORIGINAL PAPER

Quantitative Analysis of phytohormone dynamics during the induction phase of adventitious root formation in woody plants with varying rooting capacities

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Abstract
Adventitious root (AR) formation is a crucial process in vegetative propagation, influenced by endogenous hormonal signaling and species-specific physiological responses. This study investigates the dynamics of key phytohormones—indole-3-acetic acid (IAA), its inactive conjugate IAA-Asp, chlorinated auxin 4-Cl-IAA, jasmonic acid (JA), and its active form JA-Ile—during the induction phase of AR development in three woody plants with differing rooting capacities: *Prunus subhirtella* 'Autumnalis' (easy-to-root), *Castanea crenata* × *Castanea sativa* 'Marsol' (intermediate), and *Castanea sativa* 'Kozjak' (difficult-to-root). Leafy cuttings were monitored at five time points (0 min, 30 min, 1 h, 4 h, 24 h) after severance for hormone profiling via HPLC-MS/MS. Rooting performance was assessed at the end of the growing season. Results revealed genotype-specific hormonal profiles and temporal regulation patterns. Successful rooting correlated with a transient IAA peak and rapid attenuation of JA and JA-Ile signals. Conversely, high early auxin levels in *C. sativa* 'Kozjak' were accompanied by increased IAA-Asp and poor rooting outcomes. Principal component analysis underscored distinct hormone interactions across genotypes. These findings highlight the importance of hormone timing, concentration, and conjugation in AR formation and provide insights for improving propagation protocols in woody plants.

Keywords Phytohormone dynamics · Adventitious root formation · Leafy cuttings · Chestnut · Ornamental cherry · HPLC MS/MS

Introduction

Vegetative propagation is a form of asexual reproduction in plants that relies on the use of vegetative organs such as shoots, leaves, stems, or roots to produce new individuals. Because it does not involve genetic recombination, all characteristics of the mother plant are preserved and passed on to the offspring (Awotedu et al. 2021). This process depends on the presence of meristematic, undifferentiated cells that retain the capacity to differentiate into the various organs required to regenerate a complete plant (Awotedu et al. 2021). Vegetative plant propagation relies on several major methods, including cuttings, layering, grafting, and micropropagation (Yadav et al. 2018; Awotedu et al. 2021; Sarun 2026). Propagation by cuttings is the most economical method for the mass production of horticultural and forest plants while maintaining the desired genetic traits (Guan et al. 2020). The best known propagation technique is stem cuttings, which come in many forms, but the two main groups are leafy softwood cuttings and hardwood cuttings. Leafy cuttings are harvested from relatively unlignified young shoots and their rooting depends on the photosynthates produced in the propagation bed (Leakey 2004; Essola et al. 2017; Awotedu et al. 2021), in contrast, hardwood cuttings are obtained from mature, dormant stems that are over a year old (Agbo and Obi 2006). An essential step

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in vegetative propagation is the formation of adventitious roots (Chen et al. 2024). The formation of adventitious roots can be divided into three phases after the start of propagation: Induction (0–24 h), Initiation (24–72 h) and Extension (after 72 h), which lead to new visible root systems (Nag et al. 2001; Guan et al. 2020).

AR formation begins with a series of founder cell divisions and elongation of the interfascicular cambium adjacent to vascular tissues. This process is associated with a relatively high indole-3-acetic acid (IAA) content and hydrolysis of starch grains (Guan et al. 2020). Auxin, which is most abundant in nature in the form of IAA, certainly plays a central role in the early stages of adventitious root formation, although high auxin concentrations are required for optimal root formation in the induction phase (Osterc et al. 2016). Cellular IAA homeostasis is maintained via local metabolism (biosynthesis, degradation, conjugation) and transport. Intracellular free IAA levels depend on passive membrane diffusion and active polar auxin transport (PAT), driven primarily by PIN-FORMED (PIN) efflux carriers (Balzan et al. 2014). The TIR1/AFB auxin signaling pathway comprises three main protein families: TIR1/AFB F-box co-receptors, AUXIN RESPONSE FACTOR (ARF) transcription factors, and AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors (Strader and Powers 2016). When auxin levels are low, Aux/IAA repressors dimerize with ARFs, blocking their activity and preventing the expression of auxin-responsive genes. When auxin concentrations rise, the TIR1/AFB receptor binds to Aux/IAA, targeting it for ubiquitination and degradation by the 26 S proteasome. This releases the bound ARFs (ARF5 and ARF8), triggering the activation of auxin-responsive gene expression (Li 2021; Gao et al. 2024).

Plant species differ markedly in their capacity for adventitious root formation, ranging from easy-to-root to highly recalcitrant types. Endogenous auxin accumulation at the basal region of cuttings is crucial for the induction of adventitious root (AR) formation in easy-to-root species. In contrast, exogenous auxin application is often essential to stimulate AR formation in difficult-to-root species. One contributing factor is that cuttings from difficult-to-root plants tend to metabolize indole-3-acetic acid (IAA) more rapidly than those from easy-to-root plants, leading to lower levels of free IAA at the rooting site (Ford et al. 2002; Gonin et al. 2019). Previous studies (Negishi et al. 2014; Kunc et al. 2024a, b) comparing easy- and difficult-to-root species demonstrated that easy-rooting genotypes possess higher endogenous levels of indole-3-acetic acid (IAA). The effect of IAA in the plant in relation to the induction of root development is associated with a very short period immediately after the start of propagation.

Auxins are necessary for the development of the AR system, but they do not act independently. The development of ARs is largely controlled by the interplay between auxins and other plant growth regulators. In addition to auxins, jasmonic acid (JA) is also an important phytohormone for AR development (Kunc et al. 2024a; Adem et al. 2024). Currently, it is hypothesized that jasmonic acid (JA) functions as a wound-induced signal that promotes auxin biosynthesis to initiate adventitious root formation. Consequently, the tightly regulated levels and spatial distribution of JA may play a critical role in controlling the adventitious rooting response (Pan et al. 2021).

Jasmonic acid (JA) plays dual roles in adventitious rooting. It inhibits adventitious root formation and acts downstream of auxin (IAA) metabolism via GH3 family proteins in *Arabidopsis thaliana*. The molecular link between IAA and JA involves the interplay of auxin-responsive transcription factors ARF6, ARF8, and ARF17 and their target genes GH3.3, GH3.5, GH3.6, and GH3.11. ARF6 and ARF8 function as positive regulators of GH3.3, GH3.5, and GH3.6, while ARF17 acts as a negative regulator. Among these, GH3.11 (also known as JASMONIC ACID RESISTANT1, or JAR1) plays an inhibitory role in AR formation by conjugating JA to isoleucine, forming the bioactive JA-Ile. JA-Ile activates the JA receptor CORONATINE INSENSITIVE1 (COI1), which facilitates interaction with its co-receptor JAZ, a transcriptional repressor of JA-responsive genes, ultimately suppressing AR development. In contrast, GH3.3, GH3.5, and GH3.6 catalyze the conjugation of JA to amino acids such as Asp, Met, and Trp, forming inactive JA derivatives (JA-Asp, JA-Met, JA-Trp), thereby attenuating JA signaling. This highlights a regulatory balance, where auxin promotes AR formation by activating ARF6 and ARF8, which in turn favor GH3-mediated JA inactivation and suppress the COI1-JAZ pathway (Gutierrez et al. 2012; Li et al. 2020).

Prunus subhirtella Miq. 'Autumnalis' is an ornamental cherry widely used in horticulture and is generally regarded as an easy-to-root species, whereas adventitious root formation in *Castanea* species is often limited due to their inherently poor rooting capacity. (Kunc et al. 2024b, c). Numerous authors have reported the inherent difficulty of inducing root formation in *Castanea sativa* Mill. under both in vivo and in vitro conditions. As a result, hybrids such as *Castanea crenata* Siebold & Zucc. × *Castanea sativa* Mill. increasingly employed due to their better rooting capacity and enhanced tolerance to common diseases (Kunc et al. 2024b).

The aim of this study was to investigate the dynamics of key phytohormones and their metabolites during adventitious root formation in three woody plant species with differing rooting capacities. Specifically, we tracked the active

auxins (IAA and 4-Cl-IAA), the auxin catabolite (IAA-Asp), and the jasmonate signaling module (JA and JA-Ile) during adventitious root formation in three woody plant species with differing rooting capacities. Rooting parameters, including the percentage of successfully rooted cuttings, the number of main roots, and the extent of callus formation, were evaluated. The objective was to compare these morphological traits among the studied species and discuss them in relation to their respective hormonal profiles, particularly focusing on the differences between easy- and difficult-to-root genotypes. Furthermore, understanding phytohormonal dynamics during adventitious root formation in genotypes with differing rooting capacities provides crucial practical insights for optimizing propagation protocols and improving clonal success rates in woody species.

Materials and methods

Sample preparation

Leafy cuttings were collected from three woody plant genotypes with differing rooting capacities: the Japanese ornamental cherry *Prunus subhirtella* Miq. 'Autumnalis'; the Euro-Japanese chestnut commercial cultivar 'Marsol' (*Castanea crenata* Siebold & Zucc. × *Castanea sativa* Mill.); and the European chestnut native accession 'Kozjak' (*Castanea sativa* Mill.). Cuttings were taken from the current year's shoots of stock plants during the active growth phase in June 2023. The apical portions of the shoots were trimmed to a length of 12 cm. The lower 2–3 leaves were removed, and the cuttings were inserted into a suitable rooting substrate within a propagation bed. For phytohormone analysis, samples were harvested from the propagation bed at defined time intervals: 0 min, 30 min, 1 h, 4 h, and 24 h after insertion. Following collection, the cuttings were gently rinsed to remove any residual substrate and immediately flash-frozen in liquid nitrogen. For each sample, the basal portion of the cutting extending from the excision site to the first node was isolated for the analysis of phytohormones (IAA, IAA-Asp, 4-Cl-IAA, JA, and JA-Ile). These tissue segments represent the wound site where the cutting was detached from the stock plant. All harvested tissues were freeze-dried and stored at −20 °C until further analysis. To assess the rooting success, including parameters such as the percentage of rooted cuttings, number of main roots, and extent of callus formation, samples from each genotype were left undisturbed in the propagation bed until the end of the growing season, which occurred in early October. At the end of the growing season, the cuttings were carefully dug up from the propagation bed, and rooting success was evaluated by assessing the number of cuttings with adventitious roots, the number of roots formed, and the presence of callus tissue.

Experimental design

The experiment was conducted in a greenhouse at the Biotechnical Faculty of the University of Ljubljana (Ljubljana, Slovenia; 46°3′4″ N, 14°30′18″ E). The greenhouse was equipped with an automatically controlled high-pressure fogging system (Plantog, Fischamend, Austria), which maintained a relative humidity of 98–100% (Fig. S1). This high humidity prevented excessive transpiration and minimized leaf desiccation, thereby supporting the stability and viability of the cuttings.

The propagation substrate consisted of a 1:1 (v/v) mixture of peat and sand, enriched with a controlled-release fertilizer (Osmocote Exact, ICL; NPK 16-9-12 + 2 MgO + trace elements). The fogging system operated daily from 08:00 to 20:00, utilizing automated cycles that adjusted dynamically to weather conditions. On sunny days, the system maintained a cycle of 40 s of fogging followed by a 1-minute pause. Conversely, on cloudy or rainy days, the pause between fogging events was extended to 3 min. The system was completely deactivated during the night.

The experiment was structured as a 5 × 3 factorial design arranged in a Completely Randomized Design (CRD). The two investigated factors were time after severance from the stock plant (0 min, 30 min, 1 h, 4 h, and 24 h) and genotype (*Prunus subhirtella* 'Autumnalis', *Castanea crenata* × *Castanea sativa* 'Marsol', and *Castanea sativa* 'Kozjak'). The combination of these factors resulted in 15 distinct treatments. The propagation bed was divided into 15 plots (each 40 × 40 cm), which were randomly assigned to one of the 15 treatments. Each plot contained ten replicate cuttings ($n = 10$), resulting in a total sample size of 150 cuttings.

To evaluate rooting success, an additional 15 cuttings were included—one per plot. These were assessed at the end of the growing season for each genotype ($n = 5$). The distribution of treatment plots within the propagation bed was determined using simple random sampling to avoid positional bias.

Phytohormone extraction and analyses

Auxins and jasmonates were extracted according to the modified method of Almeida Trapp et al. (2014). The samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. Subsequently, 100 mg of the sample was extracted with 1 mL of isopropanol. The samples were shaken on ice for 30 min, using a shaker (Unimax 1010 Shaker, Heidolph, Schwabach, Germany), placed in a cooled ultrasonic bath (0 °C) for 1 h, and then centrifuged

at 12,000 × g and 4 °C for 10 min (5810 R; Eppendorf, Hamburg, Germany). The supernatant was transferred to a new 1.5 microcentrifuge tube and dried in a Speed-Vac (Eppendorf Austria GmbH). After drying, 200 µL MeOH was added to each sample, homogenized under vortex and centrifuged at 12,000 × g and 4° C for 10 min. The supernatant was transferred to a vial and stored at -80 °C until further analysis.

The phytohormones were analysed on an UHPLC system (Dionex UltiMate 3000; Thermo Scientific, Waltham, MA, USA) coupled with a tandem mass spectrometer (LTQ XL; Thermo Scientific, Waltham, MA, USA) following the modificated method Almeida Trapp et al. (2014). The C18 column (150 × 4.60 mm; 3 µm column; Gemini; Phenomenex, Torrance, CA, USA) was used to separate the jasmonates and auxins temperature was operated at 25 °C. The solvents used for the analysis were: solvent A, 0.1% formic acid with 3% acetonitrile in double-distilled water (v/v/v); and solvent B, 0.1% formic acid with 3% double-distilled water in acetonitrile (v/v/v). The elution profile for auxins and jasmonates was: 0–10 min, 42–55% B; 10–13 min, 55–100% B; 13–15 min 100% B; 15.1–20 min 42% B and at 20–23 min, the reconditioning of the column was performed. The mobile phase flow rate was 0.6 mL min^{-1}. Injection volume was 30 µL. The identification of the phytohormones was achieved by coupling of the UHPLC system with tandem mass spectrometer (LTQ XL; Thermo Scientific, Waltham, MA, USA), using heated electrospray ionisation. The mass spectrometer was operated in negative ion mode for identification of auxins and jasmonates. The following parameters were used for the identification of auxins: capillary temperature 200 °C; capillary voltage 8.0 V; sheath temperature 200 °C; sheath gas 30 arb; ion spray voltage 4.5 kV; tube lens − 82 V; and auxiliary gas 10 arb. While the following parameters were used for the identification of jasmonates: capillary temperature 300 °C; capillary voltage 8.0 V; sheath temperature 300 °C; sheath gas 75 arb; ion spray voltage 4.5 kV; tube lens − 82.32 V; and auxiliary gas 20 arb. To ensure high analytical selectivity within the complex plant matrix, selected reaction moritoring (SRM) transitions were monitored (Table 1). This dual-stage mass filtering approach distinguishes target phytohormones from co-extracted metabolites based on both their intact molecular mass and their unique structural fragmentation pattern. Individual phytohormones were quantified against external standards.

Chemicals

The following standards were used to identify and quantify the phytohormones: Indole-3-acetic acid, jasmonic acid, Iso-jasmonoyl-L-isoleucine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), Indole-3-acetyl-L-aspartic acid, 4-chloro-indole acetic acid (Biosynth Ltd, United Kingdom). For extraction and further analysis isopropanol and methanol (Merck KGaA, Darmstadt, Germany), formic acid (Kemika d.d., Zagreb), acetonitrile (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were used.

Statistical analysis

Data were collected using Microsoft Excel 2016 and statistically analyzed using R Commander (package Rcmdr) version 4.3.3. Two-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test for pairwise multiple comparisons to determine significant differences between groups. For multivariate data analysis, hierarchical clustering and heatmap visualization were performed using the pheatmap package in R (R Core Team, 2026). To account for differences in concentration scales across individual phytohormones, data were normalized via column-wise scaling (Z-score transformation) prior to clustering. All data are presented as means ± standard errors (SE). Statistical significance was determined at a 95% confidence level, with p-values less than 0.05 considered significant.

Results

Dynamics of phytohormones

A two-way ANOVA revealed that both the main effects of Genotype and Time, as well as their interaction (Time × Genotype), were statistically significant for all five

Table 1 SRM transitions for phytohormone quantifications

Phytohormone	Retention time	Pseudomolecular ions (m/z)		Fragmentation pattern
	(min)	$[M-H]^-$	$[M+H]^+$	(relative peak intensity %)
IAA	5.50	174	/	130 (100)
IAA-Asp	4.64	289	/	130 (100), 173 (90.30), 245 (27.29), 271 (17.78)
4-Cl-IAA	6.56	208	/	164 (100), 91 (34.67)
JA	7.76	209	/	165 (100), 191 (7.84)
JA-Ile	9.66	322	/	129.5 (100)

IAA – Indole-3-acetic acid; IAA-Asp – Indole-3-acetyaspartic acid; 4-Cl-IAA: 4-chloro-indole acetic acid; JA- Jasmonic acid; Ja-Ile: Iso-jasmonoyl-L-isoleucine

phytohormones (4-Cl-IAA, IAA, IAA-Asp, JA, and JA-Ile) (Table S1). IAA dynamics revealed two contrasting patterns among the studied genotypes. For *C. sativa* 'Marsol' and *P. subhirtella* 'Autumnalis', IAA levels remained unchanged from 0 to 1 h, peaked significantly at 4 h, and dropped back to baseline by 24 h. In contrast, *C. sativa* 'Kozjak' displayed a reverse trajectory. IAA levels were highest immediately after cutting (0 min to 1 h) and significantly declined after 4 h (Fig. 1).

The dynamics of IAA-Asp content varied substantially among the investigated genotypes, with no uniform trend observed across the time course. In *Prunus subhirtella* 'Autumnalis', a pronounced and significant peak occurred at 1 h post-severance (group 'c'), followed by a rapid decline at 4 h and a return to baseline levels by 24 h. In contrast, such a distinct early surge was absent in the remaining two genotypes.

For *Castanea crenata* × *C. sativa* 'Marsol', IAA-Asp levels remained highly stable throughout most of the experiment, showing no significant differences among the 1 h, 4 h, and 24 h time points (all grouped as 'a'). While the initial 0 min measurement was intermediate (group 'ab'), a minor, temporary fluctuation was detected at 30 min (group 'b'), which differed significantly from the later time points but not from 0 min.

Lastly, *Castanea sativa* 'Kozjak' exhibited a progressive accumulation of IAA-Asp over time. The lowest concentrations were recorded at 0 min (group 'a'), followed by a significant increase at 30 min (group 'b') and a transitional phase at 1 h (group 'ab'). A distinct maximum peak was reached at 4 h (group 'c'). By 24 h, IAA-Asp levels decreased slightly but remained elevated compared to the initial state, falling into group 'bc' (Fig. 2).

The dynamics of 4-Cl-IAA content were highly genotype-dependent (Fig. 3). In *P. subhirtella* 'Autumnalis', levels were highest immediately after severance (group 'c'), decreased significantly after 1 h (group 'b'), and continued a downward trajectory through 24 h (group 'a'). Conversely, *C. crenata* × *C. sativa* 'Marsol' exhibited the opposite pattern, where initially low concentrations (group 'a') increased significantly by 24 h (group 'b'). In *C. sativa* 'Kozjak', a distinct peak emerged at 30 min (group 'c'), which was significantly higher than at 0 min (group 'ab') and at 4 h and 24 h (group 'a'). Levels at 1 h (group 'bc') were intermediate, signaling a gradual decline after the initial 30 min surge.

A similar trend in JA content dynamics is observed in *Castanea crenata* × *C. sativa* 'Marsol' and *Prunus subhirtella* 'Autumnalis'. In *Prunus subhirtella* 'Autumnalis', a significant increase in JA content is detected at 30 minutes and 1 hour after severance, grouped as 'b', followed by a return to baseline levels by 4 hours ('a'). In *Castanea crenata* × *C. sativa* 'Marsol', JA levels also rise at 30 minutes ('c'), but by 1 hour they are no longer significantly different from either the initial or 30-minute levels. A significant decrease is observed at 4 hours, which continues through 24 hours post-severance (labeled 'a'). In *C. sativa*, JA content is highest immediately after severance ('c') and significantly decreases by 4 hours ('a'). Unlike the other two genotypes, no strong increase in JA content is observed at early time points (Fig. 4).

The dynamics of JA-Ile content exhibited highly genotype-specific, time-dependent fluctuations across the

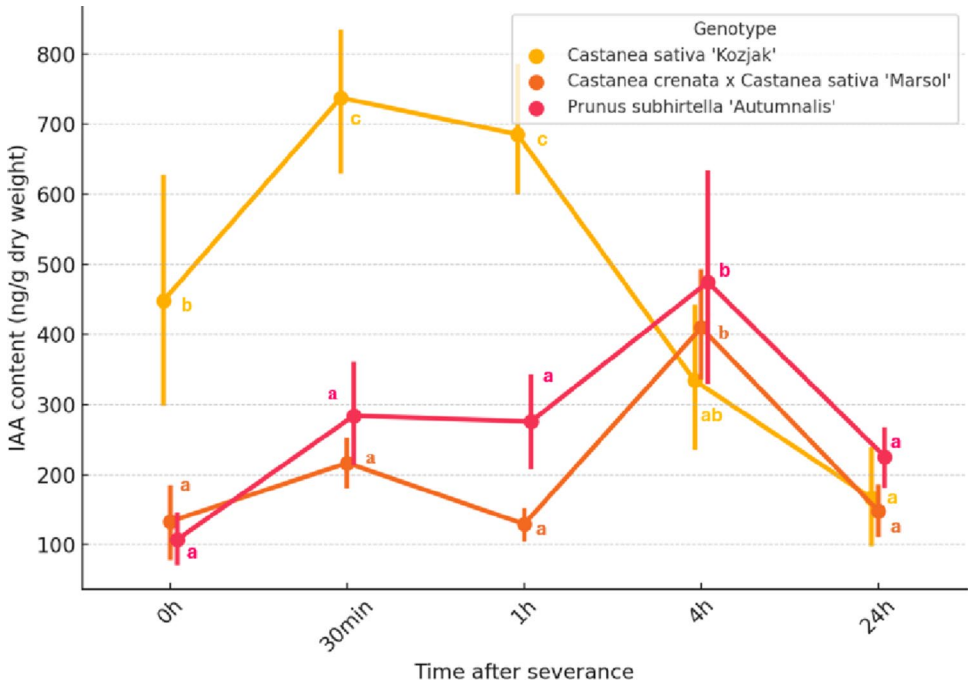


Fig. 1 Dynamics of IAA content (ng/g dry weight) over time following severance in *Castanea sativa* 'Kozjak', *Castanea crenata* × *C. sativa* 'Marsol', and *Prunus subhirtella* 'Autumnalis'. Values represent means ± standard error ($n = 10$). Different letters of the same colour indicate statistically significant differences within genotypes according to Tukey's HSD post hoc test following one-way ANOVA ($p < 0.05$)

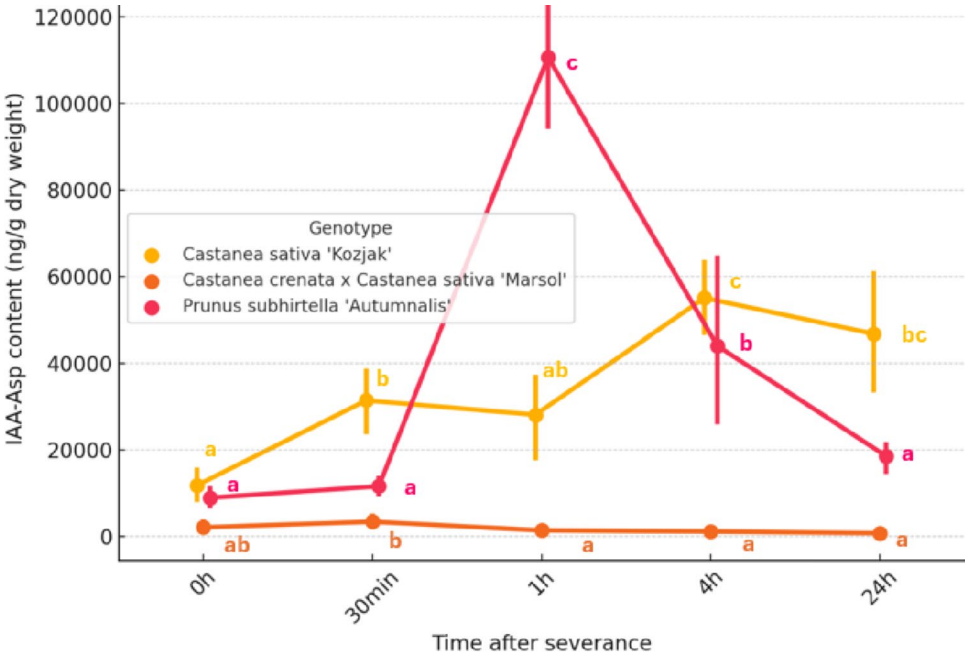


Fig. 2 Dynamics of IAA-Asp (ng/g dry weight) over time following severance in *Castanea sativa* 'Kozjak', *Castanea crenata* × *C. sativa* 'Marsol', and *Prunus subhirtella* 'Autumnalis'. Values represent means ± standard error ($n = 10$). Different letters of the same colour indicate statistically significant differences within genotypes according to Tukey's HSD post hoc test following one-way ANOVA ($p < 0.05$)

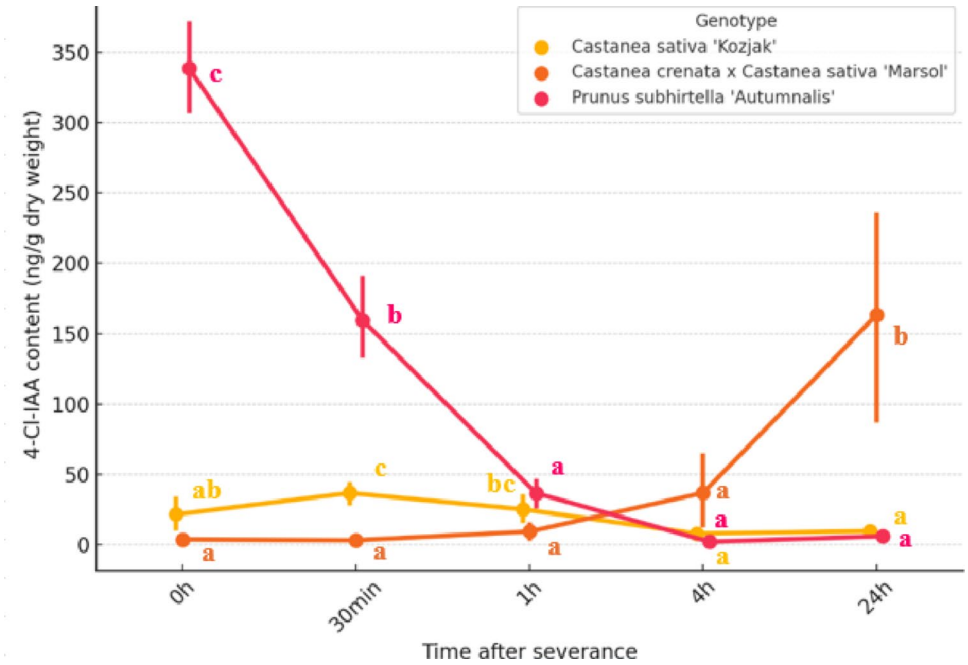


Fig. 3 Dynamics of 4-Cl-IAA (ng/g dry weight) over time following severance in *Castanea sativa* 'Kozjak', *Castanea crenata* × *C. sativa* 'Marsol', and *Prunus subhirtella* 'Autumnalis'. Values represent means ± standard error ($n = 10$). Different letters of the same colour indicate statistically significant differences within genotypes according to Tukey's HSD post hoc test following one-way ANOVA ($p < 0.05$)

investigated period (Fig. 5). In *Prunus subhirtella* 'Autumnalis', JA-Ile levels increased significantly at 30 min post-severance compared to the initial baseline (group 'd'). This was followed by a decrease at 1 h (group 'c') and a return to baseline levels by 4 h (group 'b'). By 24 h post-severance, JA-Ile concentrations fell significantly below the initial starting values (group 'a').

A similar early accumulation was observed in *Castanea sativa* 'Kozjak', where JA-Ile content peaked at 30 min and remained at this maximum level through 1 h (both classified as group 'b'). By 4 h post-severance, JA-Ile concentrations had declined back to their baseline values (group 'a').

For *Castanea crenata* × *C. sativa* 'Marsol', post-hoc analysis revealed a slightly shifted trajectory. The JA-Ile levels at 0 min and 30 min showed no significant differences (both grouped as 'ab'). A significant increase occurred at 1 h, representing the maximum peak (group 'b'), after which levels dropped significantly by 4 h and 24 h, returning to the baseline state (both grouped as 'a').

The hierarchical clustering heatmap reveals distinct, genotype and time specific trends in the relative abundance of

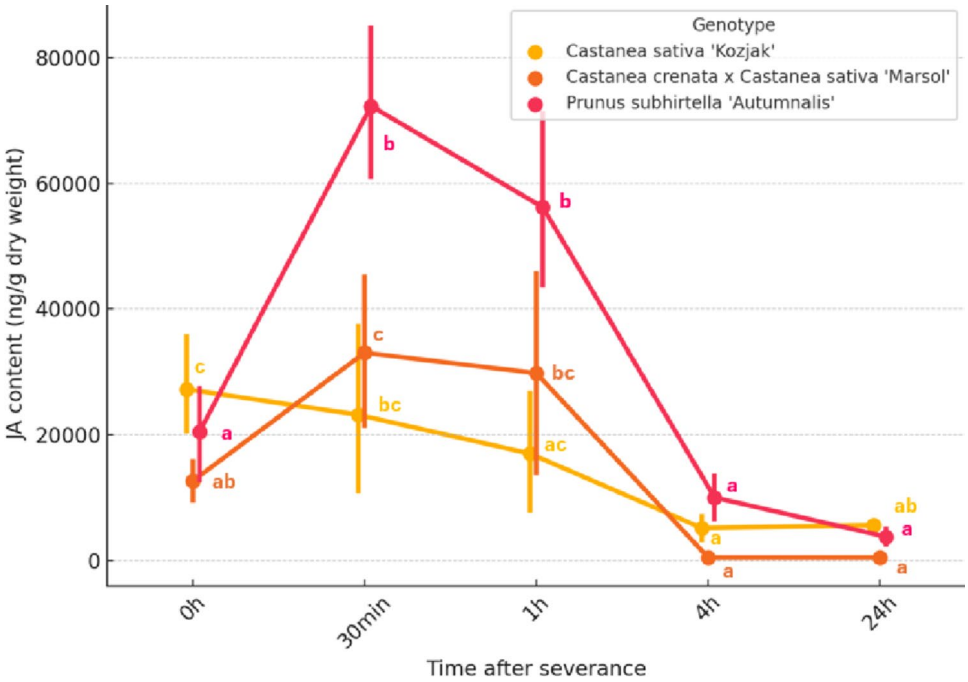


Fig. 4 Dynamics of JA content (ng/g dry weight) over time following severance in *Castanea sativa* 'Kozjak', *Castanea crenata* × *C. sativa* 'Marsol', and *Prunus subhirtella* 'Autumnalis'. Values represent means ± standard error ($n = 10$). Different letters of the same colour indicate statistically significant differences within genotypes according to Tukey's HSD post hoc test following one-way ANOVA ($p < 0.05$)

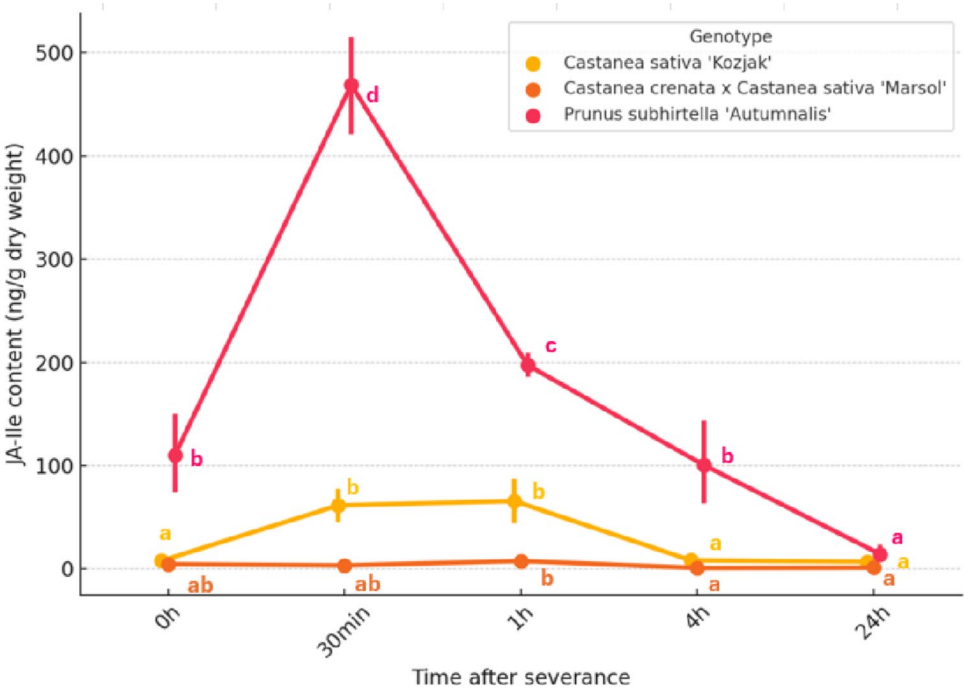


Fig. 5 Dynamics of JA-Ile content (ng/g dry weight) over time following severance in *Castanea sativa* 'Kozjak', *Castanea crenata* × *C. sativa* 'Marsol', and *Prunus subhirtella* 'Autumnalis'. Values represent means ± standard error ($n = 10$). Different letters of the same colour indicate statistically significant differences within genotypes according to Tukey's HSD post hoc test following one-way ANOVA ($p < 0.05$)

the analyzed phytohormones. Free IAA levels peaked markedly in *Castanea sativa* 'Kozjak' at 30 min and 1 h post severance, with above average levels also observed in the 'Kozjak' 0 h, as well as in *Prunus subhirtella* 'Autumnalis' and *C. crenata* × *C. sativa* 'Marsol' at 4 h.

Conversely, the 'Autumnalis' 1 h treatment stood out with the highest relative content of the auxin conjugate IAA-Asp across all 15 experimental treatments. Elevated relative levels of IAA-Asp were also detected at later time points, specifically in 'Kozjak' at 4 h and 24 h, and 'Autumnalis' at 24 h.

Regarding the chlorinated auxin variant, 4-Cl-IAA, the 'Autumnalis' 0 h control exhibited a pronounced and unique baseline elevation, achieving the absolute highest relative value in the study, while above-average levels were also noted in 'Autumnalis' at 30 min and 'Marsol' at 24 h.

Jasmonate dynamics indicated that JA and JA-Ile were most heavily overrepresented in the 'Autumnalis' genotype,

where they reached a sharp maximum at 30 min and 1 h post-severance. Additionally, above-average JA accumulation was also evident in the early response phases (30 min and 1 h) of the 'Marsol' genotype (Fig. 6).

Rooting parameters

Significant differences in rooting parameters were observed among the genotypes. Cuttings of *Prunus subhirtella* 'Autumnalis' (52.00 ± 5.86) and *Castanea crenata* × *C. sativa* 'Marsol' (33.32 ± 8.95) showed a statistically significantly higher percentage of successfully rooted cuttings compared to *Castanea sativa* 'Kozjak' (6.66 ± 3.65) (Figs. 7a and 8a and b). Callus formation was most frequent in cuttings of *Castanea sativa* 'Kozjak' (88.00 ± 3.27) (Fig. 8c), while a notably lower percentage of cuttings with callus formation was observed in *Castanea crenata* × *C. sativa* 'Marsol' (42.67 ± 5.81). Callus formation was least frequent in cuttings of *Prunus subhirtella* 'Autumnalis' (8.00 ± 8.00) (Fig. 7a). The average number of main roots was highest in cuttings of *Prunus subhirtella* 'Autumnalis' (6.18 ± 0.57), somewhat lower in *Castanea crenata* × *C. sativa* 'Marsol' (2.40 ± 0.43), and lowest in cuttings of *Castanea sativa* 'Kozjak' (0.60 ± 0.24) (Fig. 7b).

Discussion

Auxin metabolism and its role in adventitious root formation

The development of adventitious roots is a complex process that is influenced by numerous endogenous and environmental factors (Geiss et al. 2009). Phytohormones are undoubtedly central to the complex network of factors that regulate adventitious root (AR) formation (da Costa et al. 2013). Different plant species exhibit varying capacities for adventitious root formation, which are influenced by

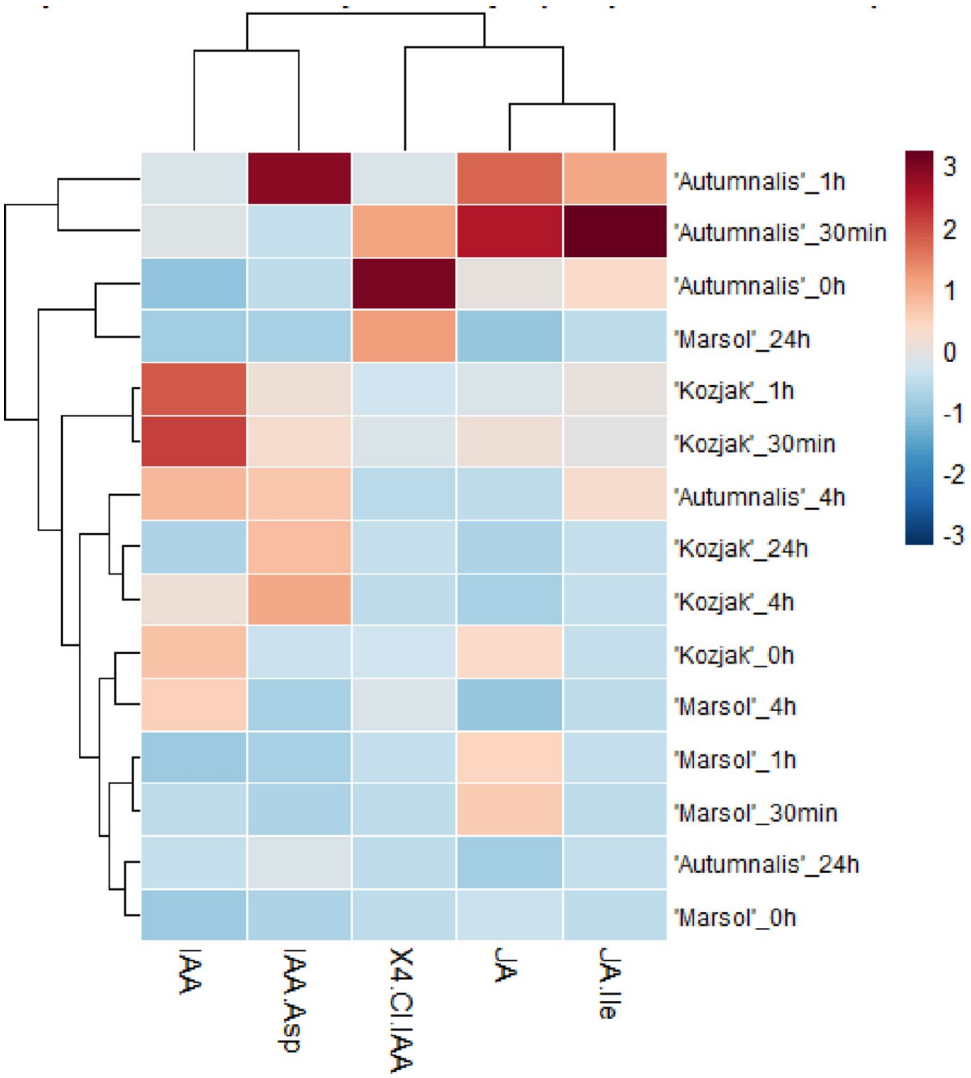


Fig. 6 Heatmap analysis representing the normalized (Z-score) levels of jasmonates (JA, JA-Ile) and auxins (IAA, IAA-Asp, 4-Cl-IAA) in *Prunus subhirtella* 'Autumnalis', *Castanea sativa* 'Kozjak', and *C. crenata* × *C. sativa* 'Marsol' at various time points after severance

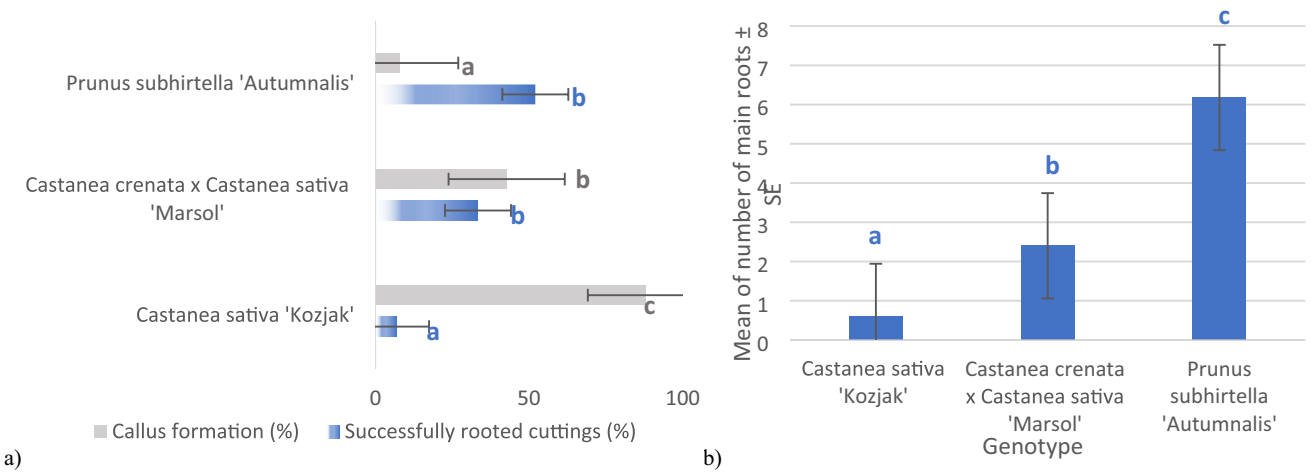


Fig. 7 Rooting outcome (**a**) Percentage of callus formation and successfully rooted cuttings (±SE) in *Castanea sativa* 'Kozjak', *Castanea crenata* × *C. sativa* 'Marsol', and *Prunus subhirtella* 'Autumnalis'. (**b**) Mean of number of main roots (±SE) observed in *Castanea sativa* 'Kozjak', *Castanea crenata* × *C. sativa* 'Marsol', and *Prunus subhirtella* 'Autumnalis'. Different letters indicate statistically significant differences among genotypes (Tukey's HSD test following one-way ANOVA, $p<0.05$; $n=10$). **b**) Different letters within each response variable indicate statistically significant differences among genotypes (Tukey's HSD test following one-way ANOVA, $p<0.05$; $n=10$)

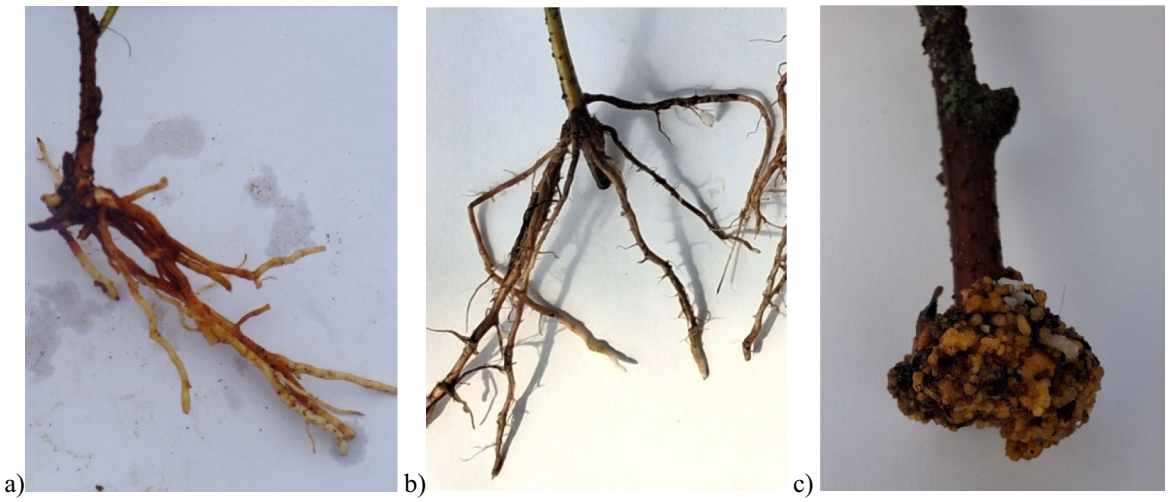


Fig. 8 Rooting parameters **a**) successfully rooted cutting of *Prunus subhirtella* 'Autumnalis' **b**) successfully rooted cutting of *Castanea crenata* × *Castanea sativa* 'Marsol' **c**) Callus formation in cuttings of *Castanea sativa* 'Kozjak'

genetic, physiological, and hormonal factors(Leakey 1985; Ford et al. 2001; Tutu et al. 2023). Our results also show the differences in rooting capacity and quality of newly formed adventitious roots between *Castanea sativa* 'Kozjak', *Castanea crenata* × *C. sativa* 'Marsol' and *Prunus subhirtella* 'Autumnalis'. *P. subhirtella* 'Autumnalis' showed the highest rooting capacity and the greatest number of well-developed main roots, suggesting a high intrinsic potential for vegetative propagation. Our results are consistent with previous studies indicating that *Prunus subhirtella* 'Autumnalis' is an easy-to-root genotype and exhibits high rooting success(Kunc et al. 2024a, c). In contrast, *C. sativa* 'Kozjak' exhibited a significantly lower rooting percentage and produced fewer main roots, higher incidence of callus formation. Callus formation is considered as an undesirable characteristic of a cutting, and is frequently observed in difficult to root cuttings (Kunc et al. 2024c). The hybrid genotype *C. crenata* × *C. sativa* 'Marsol' demonstrated intermediate rooting characteristics, consistent with previous findings that hybrids often exhibit improved rooting ability compared to *C. sativa* lines (Osterc et al. 2010; Song et al. 2021; Kunc et al. 2024b). AR formation is closely linked to high levels of IAA. According to Guan et al. (2020), AR formation coincides with a transient increase in endogenous free IAA, particularly during the early stages of propagation. Induction phase (0–24 hours) is marked by a pronounced rise in IAA content, with a peak typically occurring around 24 hours, signaling the transition to the

initiation phase (Nag et al. 2001). However, previous studies (Osterc et al. 2016; Kunc et al. 2024a) have shown that in *Prunus subhirtella* 'Autumnalis', the first IAA peak occurs earlier—approximately 4 hours after the start of propagation. Our findings align with these earlier observations and confirm a similar early IAA peak in *Castanea crenata* × *Castanea sativa* 'Marsol'. In contrast, this early peak was not observed in *C. sativa* 'Kozjak'. Although auxin is essential for adventitious root formation, its concentration must be tightly regulated. Higher endogenous auxin levels can often become inhibitory and its overaccumulation does not necessarily enhance rooting capacity(Ludwig-Müller 2011; Osterc et al. 2022). Our results show a peak in IAA content at 30 minutes and 1 hour after severance in *Castanea sativa* 'Kozjak'. Additionally, the initial IAA concentration in 'Kozjak' (448.66 ± 85.52 ng g^{-1}) is significantly higher compared to *Castanea crenata* × *Castanea sativa* 'Marsol' (133.32 ± 27.91 ng g^{-1}) and *Prunus subhirtella* 'Autumnalis' (107.25 ± 18.39 ng g^{-1}). A notable decrease in IAA content occurs in 'Kozjak' 4 hours after severance. These findings suggest that successful adventitious root formation depends not only on high IAA levels, but also on achieving an optimal concentration and precise temporal distribution of the hormone. IAA-Asp is an inactive storage form of IAA that can be converted back into free IAA by ILR1/ILL amidohydrolases. This conjugation is believed to play a key role in regulating the local availability of auxin in response to environmental stimuli. Notably, the metabolic flux of auxin is highly rapid, with significant shifts occurring within approximately 10 minutes. This indicates that auxin biosynthesis and inactivation processes operate in tight coordination to maintain cellular homeostasis(Hayashi et al. 2021; Hladík et al. 2023). In *Castanea crenata* × *Castanea sativa* 'Marsol', IAA-Asp content was initially low, followed by a statistically significant increase 30 minutes after severance. Subsequently, the levels declined and remained stable throughout the first 24 hours. In both *Castanea crenata* × *Castanea sativa* 'Marsol' and *Prunus subhirtella* 'Autumnalis', IAA-Asp content decreases 4 hours after severance, coinciding with a rise in free IAA levels. In contrast, in *Castanea sativa* 'Kozjak', IAA-Asp reaches its peak at 4 hours post-severance, while free IAA content declines. These results may suggest a dynamic shift between the active and inactive forms of auxin during the induction phase of adventitious root formation, potentially influencing AR development. The chlorinated auxin, 4-chloroindole-3-acetic acid (4-Cl-IAA), is present in certain higher plant species and has been shown to exhibit greater bioactivity than the predominant endogenous auxin, IAA, in some bioassays (Karcz and Burdach 2002). Previous studies have demonstrated that 4-Cl-IAA exhibits significant biological activity in stimulating adventitious root formation, suggesting a role in root developmental regulation(Katayama 2000). In our study the highest levels of 4-Cl-IAA were observed in *Prunus subhirtella* 'Autumnalis', which also demonstrated the greatest rooting capacity and root quality. Elevated concentrations were detected immediately after severance and at 30 minutes post-severance, followed by a decline. In *Castanea crenata* × *Castanea sativa* 'Marsol', which did not significantly differ from *P. subhirtella* 'Autumnalis' in the percentage of successfully rooted cuttings but exhibited differences in the number of main roots and callus formation, a marked increase in 4-Cl-IAA was recorded only after 24 hours. In contrast, *Castanea sativa* 'Kozjak' displayed lower overall 4-Cl-IAA levels and a less pronounced increase. Variations in 4-Cl-IAA content immediately after severance (0 h) among all three genotypes suggest a species-specific endogenous 4-Cl-IAA profile. The late increase in 4-Cl-IAA observed in *Castanea crenata* × *Castanea sativa* 'Marsol', which coincided with a decline in IAA levels—unlike in *Castanea sativa* 'Kozjak'—may reflect a positive auxin-related effect on AR formation. In *Castanea sativa* 'Kozjak', the highest concentrations of 4-Cl-IAA were recorded at the earliest time points, mirroring the early IAA peak within the first hour after severance. This may indicate an excessively strong auxin response immediately post-severance in *C. sativa* 'Kozjak', potentially contributing to an inhibitory effect on AR formation. The role of 4-Cl-IAA in AR formation remains largely unexplored, and to date, no studies have specifically addressed this topic.

Interplay between jasmonate and auxin dynamics during wounding and adventitious root induction

Adventitious root formation in cuttings begins with a wound-induced increase in jasmonic acid (JA) (Steffens and Rasmussen 2016). However, to activate plant defence responses, JA must first be enzymatically conjugated to isoleucine (Ile), forming the bioactive jasmonate JA-Ile. This conjugate binds to and activates the SCF^{COI1} ubiquitin ligase complex, which targets JAZ (Jasmonate ZIM-domain) proteins for degradation via the 26S proteasome. The removal of JAZ repressors releases multiple transcription factors from JAZ-mediated inhibition, thereby enabling the activation of downstream jasmonate-responsive genes and defence pathways(Pauwels and Goossens 2011; Lortzing and Steppuhn 2016). Our results indicate that JA is rapidly synthesized in response to wounding and initiates defense responses. This JA surge represents one of the earliest hormonal signals following severance. JA and JA-Ile levels are significantly lower in genotypes from the genus *Castanea*. A similar dynamics trend is observed in *Prunus subhirtella* 'Autumnalis' and *Castanea crenata* × *Castanea sativa* 'Marsol', with a significant increase in JA 30 minutes after

severance, whereas in *Castanea sativa* 'Kozjak', JA levels peak immediately after cutting and significantly decrease 4 hours after severance. Significantly higher levels of JA-Ile in *Prunus subhirtella* 'Autumnalis' may indicate more efficient plant defense responses. Although a less pronounced increase in JA-Ile is also observed in *Castanea* genotypes, the auxin content is also important for the subsequent process of AR formation. Our results may highlight the importance of the auxin peak in the induction phase of AR formation. When auxin concentration is high, the auxin receptor TIR1/AFB binds to Aux/IAA repressors. Through the action of the 26S proteasome, these AUX/IAA proteins are ubiquitinated and subsequently degraded, thereby releasing ARF6 and ARF8 transcription factors. This release allows the activation of auxin-responsive genes(Li et al. 2020; Gao et al. 2024). Among their downstream targets, GH3.3, GH3.5, and GH3.6 act as positive regulators of AR formation by catalyzing the conjugation of JA to amino acids leading to the formation of inactive JA derivatives (JA-Asp, JA-Met, JA-Trp). This process attenuates JA signaling (Gutierrez et al. 2012; Li et al. 2020). The peak in IAA content observed in *Prunus subhirtella* 'Autumnalis' and *Castanea crenata* × *Castanea sativa* 'Marsol' may indicate the conjugation of JA to amino acids by GH3.3, GH3.5, and GH3.6, resulting in the formation of inactive JA derivatives. Additionally, in these two genotypes, we observed a decline in the inactive form of IAA (IAA-Asp), JA, and JA-Ile 4 hours after severance, which are associated with negative effects on adventitious root (AR) formation. Higher levels of JA-Ile in *P. subhirtella* 'Autumnalis' may suggest enhanced plant defense responses, reducing stress and consequently leading to reduced callus formation and a greater number of main roots. In contrast, *Castanea sativa* 'Kozjak' showed high IAA levels immediately after severance, which dropped significantly after 4 hours, while the inactive form of IAA increased. Furthermore, elevated levels of JA, JA-Ile, and 4-Cl-IAA were only detected up to 4 hours post-severance, after which they significantly decreased. Notably, no pronounced surge in JA levels was observed in *Castanea sativa* 'Kozjak' immediately after severance that would typically indicate a wound response. Our results suggest that not only is the absolute concentration of IAA important, but also the timing and activation of its peak. Future research should further investigate the role of 4-Cl-IAA in AR formation and its interaction with other phytohormones.

Conclusion

This study demonstrates that the dynamics of auxin and its cofactors during the early induction phase of adventitious root formation are strongly genotype-dependent in woody plant cuttings. The successful rooting observed in *Prunus subhirtella* 'Autumnalis' and *Castanea crenata* × *C. sativa* 'Marsol' is closely associated with a transient peak in free IAA and a coordinated decline in its inactive conjugates (IAA-Asp) and jasmonate levels (JA, JA-Ile). In contrast, the poor rooting performance of *C. sativa* 'Kozjak' correlates with elevated early auxin levels, delayed or diminished hormone turnover, and increased accumulation of inactive forms. These results suggest that both the timing and magnitude of phytohormonal changes—particularly the auxin–jasmonate balance—play a central role in the rooting capacity of cuttings. Understanding these regulatory mechanisms can inform the selection of genotypes and optimization of propagation protocols for recalcitrant woody species.

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Data availability The remaining data presented in this study are available on request from the corresponding author. The remaining data are not publicly available due to privacy.

Declarations

Competing interests The authors declare no competing interests.

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