

Stock plant etiolation and immersion in IBA solution stimulate rooting capacity of *Pinus patula* cuttings

Georgina Irasema Bautista-Ojeda^{1*}, J. Jesús Vargas-Hernández^{1*}, Marcos Jiménez-Casas¹, Arnulfo Aldrete¹, María Cristina Guadalupe López-Peralta², F. Víctor Conde-Martínez³ e Hilda Araceli Zavaleta-Mancera³

¹Postgrado en Ciencias Forestales, Colegio de Postgraduados. Km. 36.5. Carr. México-Texcoco. 56264, Montecillo, Estado de México, México.

²Postgrado en Recursos Genéticos y Productividad- Genética, Colegio de Postgraduados. Km. 36.5. Carr. México-Texcoco. 56264, Montecillo, Estado de México, México.

³Postgrado en Botánica, Colegio de Postgraduados. Km. 36.5. Carr. México-Texcoco. 56264, Montecillo, Estado de México, México

*Corresponding authors: bautista.georgina@colpos.mx, +52 1 618 107 07 98; vargashj@colpos.mx, +52 1 595 114 90 20

Abstract

Cloning by rooted cuttings from individuals with low adventitious root-forming capacity depends on multiple factors such as light exposure during cutting development and immersion time in indole-3-butyric acid (IBA) solution. This study evaluated the effects of stock plant etiolation on total soluble sugars concentration and endogenous growth regulators profile, and its combined effect with exogenous IBA application on rooting capacity of *Pinus patula* cuttings. Early morpho-anatomical changes related to root formation were also assessed. A first trial evaluated immersion time of cuttings (0, 15, 30, 60, 120, and 240 s) in a 5000 ppm IBA liquid solution. Afterwards, the combined effect of stock plant etiolation (0, 3, and 6 days) with IBA application (0 and 120 s immersion) was tested. Cuttings were rooted under controlled conditions (20–24°C and 85–100 % relative humidity) for twelve weeks. Variables measured included the percentage of live cuttings, callus formation, rooted cuttings, number of primary roots, length of the longest root, average primary root length, and percentage of rooted cuttings with secondary roots. Both trials showed over 98 % survival. IBA-immersion for 120 s achieved 83.6 % rooted cuttings. In the second trial, six-day etiolation with IBA-application produced the highest rooting (64.4 %) with more and longer primary roots, and 70.7 % secondary root presence. Etiolation reduced sugar content and increased endogenous auxin levels in cuttings compared to non-etiolated ones. Changes in soluble sugars and growth regulators due to etiolation enhanced root formation. Controlling these parameters provides an operational protocol for efficient cloning of *Pinus patula*.

Keywords: adventitious roots, auxins, cloning, plant growth regulators, vegetative propagation.

Introduction

Pinus patula Schiede ex Schltdl. et Cham. is an endemic Mexican conifer, recognized for its economic importance (Rivera-Rodríguez et al. 2016). Its natural distribution is in humid environments ranging from subtropical to warm-temperate at altitudes between 1,600 and 3,100 m (Lira 2020). *Pinus patula* has been introduced commercially outside its native range in Africa, India, South America, and Australia (Lira 2020), and several operational tree breeding programs have been established for this species (Wright et al. 1996, Rivera-Rodríguez et al. 2016). Given the potential benefits of using clonal propagules from superior genotypes for commercial plantations (Park 2002, White et al. 2007), developing operational protocols for rooting *Pinus patula* cuttings is crucial.

Adventitious root (AR) formation in cuttings is a complex process. Its success depends on the physiological state and genetic traits of the stock plant, as well as on cutting handling and environmental conditions during rooting (Kanmegne et al. 2017). Factors such as etiolation (reduction or total elimination of light) and increased endogenous auxins produce anatomical and physiological changes that commonly enhance rooting percentages (Hartmann et al. 2014, Pacholczak et al. 2017).

ARs in cuttings arise from non-root tissues, i.e., meristematic cells with cambial capacity in stems (Legué et al. 2014), such as xylem parenchyma cells or other phloem elements, usually parenchymatic, which resume meristematic activity to form roots. The proximity of these tissues to sources of plant growth regulators (PGRs) and sugars is important for rooting. AR formation originates from cells near the stem's vascular cylinder (Gonin et al. 2019) or callus cells differentiating near the wound (Hamann 1998) and is considered particularly difficult in woody conifer species.

Reducing light exposure in the stock plant during cuttings development affects in two ways: 1) it promotes tissue juvenility by reducing anatomical structures such as fibers and sclerenchyma (smaller stem diameter) and prevents lignification of secondary xylem and periderm (Apine et al. 2013); 2) it increases endogenous auxins and decreases gibberellins in cuttings, promoting root primordia development (Pacholczak et al. 2005, Hartmann et al. 2014). Husen (2008) reports that etiolation of *Tectona grandis* stock plants before rooting increases total soluble sugar content due to greater starch conversion and mobilization; Agulló-Antón et al. (2014) found starch hydrolysis, sugar mobilization, and soluble sugar accumulation during root induction.

Auxins regulate all plant development processes (Hartmann et al. 2014), and their transport rate and pathway are influenced by light availability and quality (da Costa et al. 2013). Rooting occurs in three phases: induction, initiation, and elongation, which require different growth regulator concentrations and interactions, particularly the natural auxin indole-3-acetic acid (IAA) (Wei et al. 2020). Higher auxin concentration is needed during induction but reduced during elongation to avoid developmental inhibition (da Costa et al. 2013, Wei et al. 2020). Therefore, high auxin levels immediately after cutting can improve rooting (da Costa et al. 2013).

The main natural auxin is IAA (Frick & Strader 2018, Druege et al. 2019). Produced in meristematic tissues and young leaves, it regulates vascular differentiation (Aloni 2013). Another form, indole-3-butyric acid (IBA), originally described as synthetic auxin, has been reported as natural PGR (Korasick et al. 2013, Kreiser et al. 2016, Bhatla & Lal 2023), and as an IAA precursor acting via β -oxidation in peroxisomes (Strader & Bartel 2011, Druege et al. 2019). IBA produces similar effects to IAA and is more heat- and light-stable (Basuchaudhuri 2021; Harshita & Singh 2022).

Etiolation of stock plants combined with IBA on cuttings improves rooting capacity (Pacholczak et al. 2005, 2017, Apine et al. 2013), though this combination has not been evaluated in *Pinus patula*. Rivera-Rodríguez et al. (2016) found that IBA application on cuttings harvested from one-year-old stock plants achieved over 50 % rooting, while combining 18-months-old stock plants with fertilization management and 20 s immersion in IBA solution reached 73.8 % rooting (Bautista-Ojeda et al. 2022).

The aim of this study was to evaluate the effect of stock plant etiolation, combined with 5000 ppm indole-3-butyric acid (IBA) application on rooting capacity of *Pinus patula* cuttings and characterize early morpho-anatomical and physiological changes associated with AR formation, to establish a cloning protocol for juvenile stock obtained from selected mother trees in an operational commercial plantation program.

Materials and methods

Stock plant production

The study was done at the experimental forest nursery, Campus Montecillo, Colegio de Postgraduados (geographical

coordinates: 19° 27' 38.25" N, 98° 54' 23.91" W, 2,240 m altitude). Two successive rooting trials were established with stock plants aged 11 and 14 months, produced with germplasm from the second-generation *Pinus patula* seed orchard located in Aquixtla, Puebla (19° 43' 11.9" N, 97° 59' 21.3" W, 2,930 m altitude). Stock plants were grown in a greenhouse with 60 % shade netting, in individual 10 L containers, using a substrate composed of 40 % of a base mixture (15 % peat, 15 % vermiculite, and 10 % perlite) and 60 % composted pine bark. Watering was done by saturating the substrate twice a week with distilled water, or as needed by the plants.

Cutting production

To stimulate cutting formation, a fertilization routine with Peters Professional® (20-20-20 N-P-K) at 1.0 g L⁻¹ was applied every 15 days in combination with pruning. The first pruning was performed at six months of age when seedlings were approximately 15 cm tall; later, three cuts of the longest shoots were made to renew them, maintaining the hedges at about 20 to 30 cm height, following Bautista-Ojeda et al. (2022). To achieve uniformity, a maximum of five shoots per branch were left. The last pruning before preparing the cuttings was done four months prior to harvest.

Trial establishment

Cuttings for both trials were obtained from the main stem and primary branches of stock hedges, measuring 10 to 14 cm in length, with primary needles and fewer young secondary needles; shoots had light green color and an immature terminal bud. After removal of needles from its lower third, cuttings were subjected to the IBA treatment and then inserted into the substrate.

Cuttings were dipped for 15 minutes in a fungicide (Captan 1.5 g L⁻¹) solution using distilled water before applying the IBA treatments. The rooting substrate was a mixture of fresh pine sawdust and composted bark at a 90:10 (vol.) ratio, as suggested by Escamilla-Hernández et al. (2020), in a controlled-environment rooting chamber (20-24°C and 85-100 % relative humidity) to reduce vapor pressure deficit and transpiration rate, preventing cutting dehydration (Hartmann et al. 2014). Automated nebulization watering was used to maintain humidity above 85 %, with spores of a *Trichoderma* spp. strain added as a preventive measure against pathogenic fungi.

Treatments and experimental design

The first trial (*T*₁) included six treatments with different immersion times (0, 15, 30, 60, 120, and 240 s) of the cuttings' basal end in a 5000 ppm IBA solution, arranged in a randomized block design with three replicates and 20 cuttings per experimental unit. After analyzing this trial's results, the treatment with the highest rooting percentage was selected to evaluate its response, combined with the stock-plant etiolation factor in the second trial (*T*₂).

In *T*₂, the combination of stock-plant etiolation for 0, 3, and 6 days with immersion of cuttings in 5000 ppm IBA for 0 and 120 s was evaluated. To etiolate stock-plants, hedge pots were placed in a tunnel covered with black fabric to block 100 % of

light for 3 and 6 days and watered by subirrigation; non-etiolated plants were kept in the same watering conditions but uncovered. After harvest, cuttings were immersed in 5000 ppm IBA for 0 or 120 s. A factorial (3x2) experimental design was used with four replicates and 21 cuttings per experimental unit.

Evaluation of rooting response of cuttings

We evaluated the cuttings 12 weeks after establishing each trial (T_1 and T_2). Each live cutting was individually removed from the substrate to verify presence of callus and adventitious roots. A cutting was considered dead if more than 50 % of the stem was necrotic. For each experimental unit, the percentage of live cuttings (S), with callus (C), and/or with roots (R) was evaluated; root formation is considered a subsequent stage following callus formation, so rooted cuttings were grouped separately from those with only callus. Cuttings were considered rooted if they had one or more roots at least one mm long. For rooted cuttings, the number of primary roots (NPR), length of the longest root (LLR), average length of primary roots (ALR), and the percentage with secondary roots (PSR) were determined.

Early anatomical descriptions of transverse sections of the base of cuttings were made to characterize adventitious root formation in T_1 . Fragments of one cm from the basal zone (where AR formation occurs) were fixed in FAA solution (10 % formaldehyde, 50 % ethanol, and 5 % acetic acid) for 48 hours. They were then dehydrated in ascending ethanol series, embedded in paraffin following Zavaleta's protocol (1994), sectioned at 10 μ m with a rotary microtome (American Optical, USA), stained with safranin (0.5 %) and fixed green (0.12 % in ethanol), and observed using a Zeiss Axiostar plus microscope with photographs taken with an Axiocam 5 RMc camera mounted on an Axioscope 2 Plus.

Physiological evaluation of cuttings (T_2)

After etiolation (0, 3, and 6 days) total soluble sugars (TSS) content and endogenous PGR were evaluated. TSS was determined in a sample of four cuttings per etiolation treatment, with three sub-samples per cutting. Sugars were extracted using 80 % ethanol by the Soxhlet method, quantified through the sulfuric acid-anthrone method (Loewus 1952) at 625 nm with a UV-visible spectrometer (Jenway™ 6305), and expressed as mg g⁻¹ fresh weight using a glucose calibration curve.

Growth regulators (gibberellic acid (GA₃), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), abscisic acid (ABA), trans-zeatin (TZ), cis-zeatin (CZ), and kinetin (K)) were estimated in a lyophilized sample of four cuttings per etiolation level. Samples were ground, extracted following Pan et al. (2010), and analyzed via high-performance liquid chromatography (HPLC) using an Agilent 1100 system with a Zorbax SB-C8 column, applying a gradient of water with 0.1 % trifluoroacetic acid and acetonitrile with 0.1 % trifluoroacetic acid, from 22 to 50%, at 60°C, with detection at 254 nm using Sigma-Aldrich standards for calibration.

Statistical Analysis

A variance analysis was performed on the response variables using the Glimmix procedure in SAS software (SAS 9.4 2013). We used a binomial distribution with a logit link for binomial response variables (S, C, R and PSR); for NPR, LLR and ALR a Poisson distribution was assumed; and for physiological variables (sugar and growth regulator contents) a normal distribution was used. The analysis model varied depending on the experimental design and specific factors in each trial. Variables from both trials, as well as physiological responses, utilized different models—one-factor or two-factor models with interactions. When significant effects related to a factor were detected, means were compared using Tukey's test ($\alpha=0.05$) (SAS 9.4 2013).

Results

T_1 trial. Effect of immersion time in IBA solution on rooting capacity

The immersion time (0, 15, 30, 60, 120, and 240 seconds) of cuttings in a 5,000 ppm IBA solution did not affect survival or callus formation (C), but it did influence rooting percentage (R), number of primary roots (NPR), root length (LLR and ALR), and presence of secondary roots (PSR) (Table 1). After 12 weeks of establishing T_1 , survival ratio of cuttings was 99 %; presence of callus ranged from 16.3 % to 39.8 %, with no significant differences between treatments. However, immersion for 120 s caused the highest rooting percentage (83.6 %) and number of primary roots per cutting, while immersion for 240 s caused a significant decrease in rooting success (51.6 %). The 15-s immersion treatment recorded the shortest root length and the lowest percentage of cuttings with secondary root formation (Table 1), indicating that rooting was slower in this treatment or that roots developed at a slower pace compared to other treatments.

Anatomy of adventitious root formation

Figure 1 shows the anatomy of the base of *Pinus patula* cuttings at four rooting stages. The cutting at day 0 showed typical *Pinus* secondary stem structure: vascular cambium formed by thin-walled cells with active divisions, continuous secondary xylem consisting of tracheids interrupted by resin ducts, and a pith of fundamental tissue. Outside the cambium, phloem was inconspicuous with scattered idioblasts; the cortex had 10-14 parenchyma layers and numerous resin canals, with an unstratified and lignified epidermis on the outer tangential face (Figure 1A). The cutting at 12 weeks without visible callus at the base showed internal anatomical changes: highly active vascular cambium with numerous tangential divisions, secondary xylem with more cell rows, poorly recognizable secondary phloem, disorganized cortex, and cortex cells containing abundant tannins and obliteration. Beneath the epidermis, cell divisions suggesting phellogen were observed (Figure 1B). The cutting at 12 weeks with visible callus presented major anatomical changes: an indistinct structure with numerous concentrically arranged meristematic cells emerging from the vascular cambium; some phloem cells also participated. The cortex

lost resin canals; cortex cells had thickened dark walls, and the outer cortex developed periderm and suberized cells (Figure 1C). The cutting with adventitious root primordium emergence showed a little-differentiated meristematic protuberance where root meristem tissues were not yet recognizable. Cellular changes for root primordium development originated from the vascular cambium. Tissue organization was disrupted at the xylem, cambium, and phloem levels, with dividing tissue directed toward the adventitious root primordium, suggesting preparation for vascular tissue differentiation to support root growth. The epidermis was completely replaced by a periderm with numerous suber cells and tannins (Figure 1D).

***T₂* trial. Effect of etiolation on the physiological state of the cuttings.**

Etiolation of stock plants significantly affected ($P < 0.05$) TSS content (Figure 2), as well as auxin and cytokinin contents

(Table 2). Soluble sugar content decreased as etiolation period increased, from 8.50 mg g^{-1} in the non-etiolated control cuttings to 2.58 mg g^{-1} after six days of etiolation.

On the other hand, the main endogenous growth regulators found in the cuttings were auxins and cytokinins, while gibberellins were not detected (Table 2). Six days of etiolation caused an increase in total auxin concentration, mainly associated with a rise in indole-3-acetic acid (IAA). Although cytokinins showed irregular behavior in individual isoprenoid types (CZ and K), a significant reduction in total cytokinin content was observed after six days of etiolation (Table 2). An important finding was the detection of endogenous indole-3-butyric acid (IBA) in the cuttings, though at slightly lower levels than IAA and exhibiting an opposite trend in response to etiolation, with the highest concentration ($0.011 \text{ } \mu\text{g g}^{-1}$) in non-etiolated plants and equal content in plants subjected to 3 and 6 days of etiolation (Table 2).

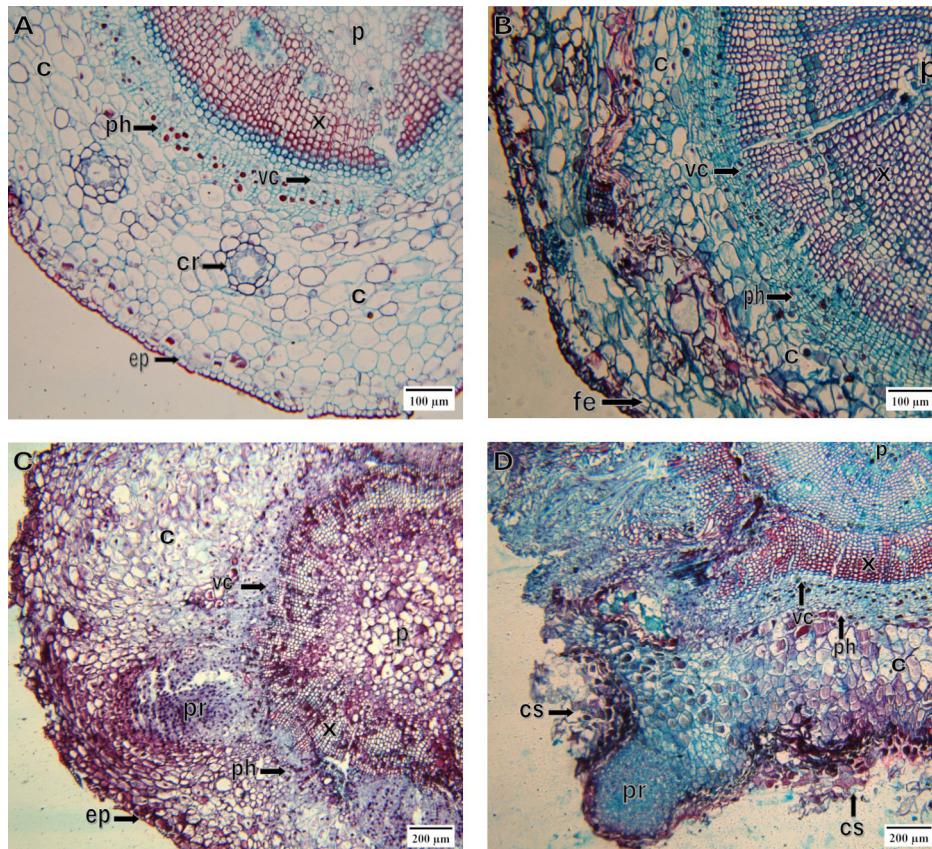


Figure 1

Anatomy of the base of *Pinus patula* cuttings at four rooting stages; A) cutting at 0 days; B) cutting at 12 weeks without visible callus; C) cutting at 12 weeks with visible callus; and D) cutting at 12 weeks with adventitious root primordium. Bright-field light microscopy, transverse section. ep, epidermis; c, cortex; cr, resin canal; ph, phloem; vc, vascular cambium; x, xylem; p, pith; pe, periderm; cs, suber cells; fe, phellogen; pr, adventitious root primordium.

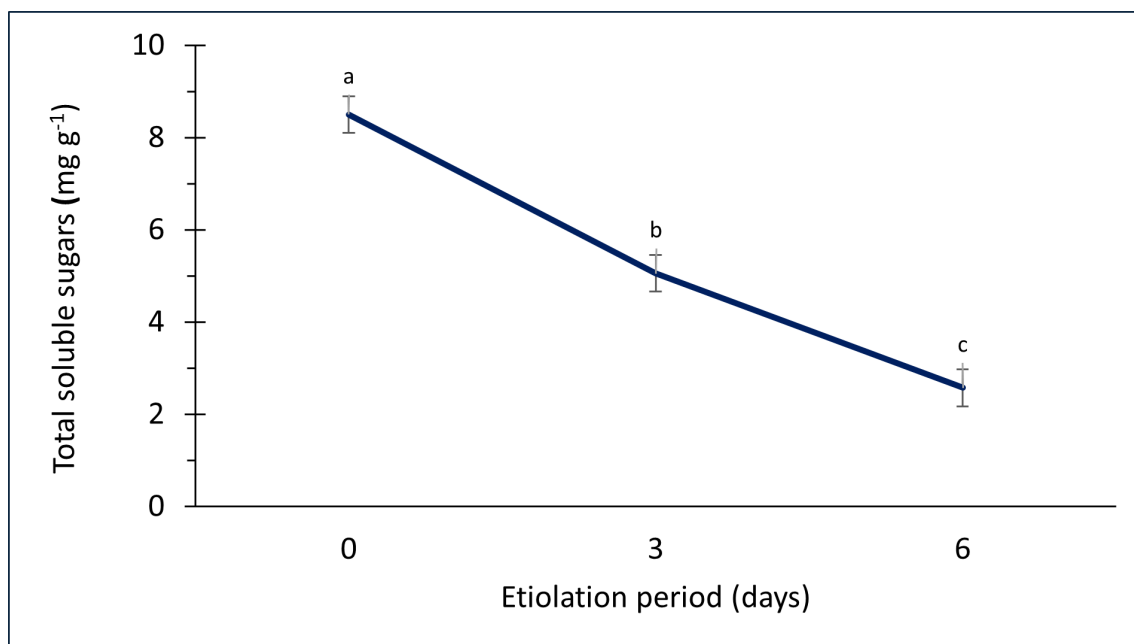


Figure 2

Total soluble sugar content in *Pinus patula* cuttings harvested from stock plants with different etiolation periods. Bars indicate standard errors. Different letters indicate statistically significant differences ($p < 0.05$).

Table 1

Average values ($\pm$ standard error) of rooting response variables by immersion time of cuttings in a 5,000 ppm IBA solution, in the T_1 rooting trial of *P. patula* cuttings.

Immersion time (s)	C (%) ¹	R (%)	NPR	LLR (cm)	ALR (cm)	PSR (%)
0	29.0 $\pm$ 6.2 a ²	69.6 $\pm$ 6.3 ab	1.9 $\pm$ 0.1 b	10.8 $\pm$ 0.9 a	8.9 $\pm$ 0.8 a	76.6 $\pm$ 7.1 a
15	26.7 $\pm$ 6.6 a	68.9 $\pm$ 7.1 ab	2.0 $\pm$ 0.1 b	7.0 $\pm$ 0.8 b	5.6 $\pm$ 0.7 b	29.7 $\pm$ 8.3 b
30	39.8 $\pm$ 6.5 a	58.5 $\pm$ 6.6 ab	2.3 $\pm$ 0.2 ab	10.3 $\pm$ 1.0 a	7.1 $\pm$ 0.8 ab	74.2 $\pm$ 9.0 a
60	17.5 $\pm$ 6.1 a	71.7 $\pm$ 5.9 ab	2.9 $\pm$ 0.2 a	8.3 $\pm$ 0.8 ab	6.1 $\pm$ 0.6 ab	59.3 $\pm$ 8.3 ab
120	16.3 $\pm$ 5.8 a	83.6 $\pm$ 5.8 a	2.9 $\pm$ 0.2 a	9.8 $\pm$ 0.8 a	7.0 $\pm$ 0.6 ab	67.6 $\pm$ 7.3 a
240	38.1 $\pm$ 6.4 a	51.6 $\pm$ 6.6 b	2.3 $\pm$ 0.2 ab	10.9 $\pm$ 1.0 a	8.3 $\pm$ 0.9 ab	69.1 $\pm$ 8.9 a

¹C: cuttings with callus; R: cuttings with roots; NPR: number of primary roots; LLR: length of longest root; ALR: average length of primary roots; PSR: cuttings with presence of secondary roots. ²Means followed by standard error. Different letters in the same column indicate statistically significant differences ($p < 0.05$).

Table 2

Average values of auxin and cytokinin content by etiolation period of stock plants in the T_2 rooting trial of *Pinus patula* cuttings.

Etiolation period (days)	Auxins ($\mu\text{g g}^{-1}$)			Cytokinins ($\mu\text{g g}^{-1}$)		
	IAA	IBA	total	CZ	K	total
0	0.014 b	0.011 a	0.025 ab	0.339 a	0.006 b	0.345 ab
3	0.011 b	0.006 b	0.017 b	0.400 a	0.014 a	0.414 a
6	0.026 a	0.006 b	0.033 a	0.264 a	0.012 ab	0.276 b

IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; CZ: cis-zeatin; K: kinetin. Different letters in the same column are statistically different ($p < 0.05$).

Effect of etiolation and IBA application on rooting

In the T_2 trial, etiolation and IBA separately affected ($p \leq 0.05$) callus (C) and root formation (R), as well as root length (LLR and ALR), with no significant interaction detected between them. However, these factors did not affect survival (S) (Tables 3 and 4). IBA also influenced the number of primary roots (NPR) and the presence of secondary roots (PSR) (Table 4). However, for most response variables, an additive (independent) effect of both factors was observed, since except for NPR, no significant interaction between them was found.

Survival rate in T_2 trial was 99 %. Etiolation of stock plants reduced the percentage of cuttings with callus formation from 70.7 % in the non-etiolated control to 48.6 % after six days of

etiolation, but increased root formation from 28.1 % to 47.3%, and average root length increased by more than two cm between these treatments. The percentage of rooted cuttings with secondary roots ranged from 34.8 % in control cuttings to 56.0 % in those from stock plants etiolated for six days (Table 3). Similarly, immersion for 120 s in a 5000 ppm IBA solution reduced callus formation percentage but more than doubled rooting percentage compared to the control, from 24.5% without IBA to an average of 54.1 %. It also increased the NPR per rooted cutting from 1.55 to 2.05 and their length by two cm compared to rooted cuttings without IBA treatment (Table 4). Secondary roots in rooted cuttings were also higher with IBA application (Table 4).

Table 3

Average values of rooting response variables by etiolation period of stock plants in the T_2 rooting trial of *Pinus patula* cuttings.

Etiolation period (days)	¹ C (%)	R (%)	LLR (cm)	ALR (cm)	PSR (%)
0	70.7 a ²	28.1 b	5.63 b	4.4 b	34.8 a
3	57.6 ab	40.3 ab	7.23 ab	6.27 ab	55.6 a
6	48.6 b	47.3 a	8.07 a	6.83 a	56.0 a

¹C: cuttings with callus; R: cuttings with roots; LLR: length of longest root; ALR: average length of primary roots; PSR: cuttings with presence of secondary roots. ² Different letters in the same column indicate statistically significant differences ($p < 0.05$).

Table 4

Average values of the rooting response variables by application time of 5,000 ppm IBA in the T_2 rooting trial of *Pinus patula* cuttings.

Application of IBA (min)	¹ C (%)	R (%)	NPR	LLR (cm)	ALR (cm)	PSR (%)
0	73.2 a ²	24.5 b	1.55 b	5.97 b	4.89 b	39.7 b
2	43.8 b	54.1 a	2.05 a	7.98 a	6.76 a	57.7 a

¹C: cuttings with callus; R: cuttings with roots; NPR: number of primary roots; LLR: length of longest root; ALR: average length of primary roots; PSR: cuttings with presence of secondary roots. ² Different letters in the same column indicate statistically significant differences ($p < 0.05$).

Discussion

Anatomy of adventitious root formation

Adventitious roots emerge from groups of cells called root initials (Evert 2006). These cells can be found in phloem, parenchyma, or other cell types (Geiss et al. 2009). In cuttings of perennial woody plants, adventitious root initials commonly originate near the central core of vascular tissues and extend outward into the young secondary phloem tissue, due to the presence of numerous layers of secondary xylem and phloem (Geiss et al. 2009). In *P. radiata*, xylem parenchyma cells and procambial cells can become meristems of adventitious roots (Pizarro & Díaz-Sala 2020). Morphological-anatomical analyses from *T₁* show that adventitious roots in *P. patula* cuttings arise from vascular cambium cells and that callus formation may be a preliminary stage for root emergence or a mechanism that facilitates adventitious root protrusion through the epidermis (Dumroese et al. 2009; Eliyahu et al. 2020).

From an anatomical perspective, callus consists of poorly differentiated meristematic cells that allow initiation of new tissues, where reorganization and lignification of vascular tissue indicate formation of root primordia (Eliyahu et al. 2020; Pizarro & Díaz-Sala 2020). This reorganization is often accompanied by a thicker periderm and intense vascular cambium activity, which promotes tissue differentiation for root growth (Geiss et al. 2009). *P. patula* cuttings in Figures 1B and 1C show similar behavior, presenting cellular reorganization for callus formation that later develops into adventitious roots.

Effect of etiolation on the physiological state of cuttings

The decrease in soluble sugars in etiolated plants (Figure 2) is basically due to photosynthesis suspension because of darkness, as well as a reduction in chlorophyll content (Zerche et al. 2016, Pacholczak et al. 2017), which lowers sugar production and transport and increases the consumption of starch reserves to maintain metabolic activity and initial root formation processes (Zerche et al. 2016). In turn, darkness or light reduction induces a hormonal and metabolic reorganization, shifting concentration and balance of key growth regulators (Apine et al. 2013).

Endogenous auxin concentration increased in etiolated plants, especially indole-3-acetic acid (IAA), associated with a significant decrease in cytokinin content (Table 2). This auxin/cytokinin ratio promotes adventitious root formation and elongation, as has been noted for other species (Druege et al. 2019, Bhatla & Lal 2023, Zhou et al. 2025). This complex interaction of PGR creates a hormonal environment favorable for cloning, although it is susceptible to variations depending on species, age, and physiological state of stock plants (Gonin et al. 2019).

Auxins, both natural and synthetic, stimulate adventitious root formation by increasing cell division and elongation at the base of cuttings (Pacurar et al., 2014). Although some reports from the mid-20th century suggested the presence of indole-3-butyric acid (IBA) as an endogenous PGR in potato (Blommert 1954), this molecule was long considered not to exist

naturally in plants. However, recent studies have recorded it as an endogenous auxin in *Arabidopsis thaliana* (Damodaran & Strader 2019), hazelnut (*Corylus*) (Kreiser et al. 2016), elm (*Ulmus*) (Kreiser et al. 2016), and maize (Ludwig-Müller 2000); additional studies have analyzed issues related to IBA biosynthesis, storage, and transport (Strader & Bartel 2011, Korasick et al. 2013, Frick & Strader 2018, Bhatla & Lal 2023). To our knowledge, this is the first study reporting endogenous IBA presence in *Pinus patula* plants.

IBA is considered a natural precursor of IAA in plants (Druege et al. 2019), and its conversion primarily occurs in peroxisomes through a β -oxidation process of fatty acids, which shortens the IBA side chain to form active IAA (Druege et al. 2019). Following this theory, the opposing trend between IAA and IBA as the etiolation period increases may result from IBA converting to IAA to meet the auxin demand needed to support plant growth in darkness (Damodaran & Strader 2019). However, it should be also considered that in some species, such as maize, IBA acts alone as an auxin and not through IAA conversion (Ludwig-Müller 2000). Studies of the biosynthetic pathways of IAA and IBA in *Pinus patula* are required.

Effect of etiolation and IBA application on rooting

There is plenty evidence on the effect of exogenous auxin application stimulating adventitious root formation (Hartmann et al. 2014, Abdel-Mohsen & Rashedy 2023); however, previous experiences differ in the optimal exposure time of cuttings to IBA solution. For example, a quick dip in IBA (5000 ppm for 10 s), combined with a fertilization routine of 5 g L⁻¹ Osmocote 15-9-12 (N-P-K) on 18-months-old *P. patula* stock plants resulted in over 70 % rooting (Bautista-Ojeda et al. 2022), but the same dipping time in a 400 ppm IBA solution allowed only 19 % rooting in *Pinus halepensis* (Riov et al. 2020). On the other hand, cutting immersion in an IBA + paclobutrazol solution for 2 s allowed 98 % rooting in *P. caribaea* cuttings (Henrique et al. 2006), and a 10 s dip in a 10 g L⁻¹ IBA solution allowed over 95 % rooting for *P. pinaster* cuttings (Majada et al. 2011). In contrast, in this work, the optimal dipping time for rooting *Pinus patula* cuttings in a 5,000 ppm IBA solution was 120 s.

Araujo-Viera de Souza et al. (2021) indicated that lower lignification increases rooting potential and root length. Although Husen (2008) reported that soluble sugar content in shoots from etiolated stock plants of *Tectona grandis* is higher than in non-etiolated plants, the opposite occurred for *P. patula*; however, none of these studies evaluated starch content, which could help explain differences in soluble sugar dynamics. Zhou et al. (2025) mention that the relationship between adventitious root formation and nutrient metabolism during the induction and elongation process is complex.

During AR formation, significant changes occur in sugars and nitrogenous compounds at the base of cuttings; respiration and consumption of soluble sugars and starch also increase, causing a reorganization to maintain metabolic activities (Druege et al. 2019, Zhou et al. 2025). These changes are related to the capacity of leaves as sugar source for AR formation at the stem base (Druege et al. 2019). In some species, a lower

soluble sugar content resulting from an etiolation period is associated with higher rooting because changes occurring in the synthesis and transport of molecules critical for the process (Pacholczak et al. 2005).

After etiolation, it is recommended that cuttings be planted under natural light conditions to increase net photosynthesis and raise sugar levels that were reduced during etiolation, supporting AR development (Pacholczak et al. 2005, Druege et al. 2019). In the present work, sugar content decreased with etiolation time; subsequently, the cuttings were placed under natural light, which allowed greater rooting in the treatment with lower soluble sugar content (Table 4). However, Druege & Kadner (2008) mention that the specific role of sugars in adventitious root formation is controversial and not fully clear, so total sugar content per se cannot explain the different capacity to form adventitious roots (Druege & Kadner 2008).

On the other hand, increased IAA also produces anatomical changes in plants, such as dedifferentiation, reduced lignification, and less sclerenchyma development. At the cellular level, it facilitates cell wall softening, stretching and degradation (Wei et al. 2020). These changes increase root primordium formation, improving rooting success (Hartmann et al. 2014, Pacholczak et al. 2017).

Auxin application activates sugar metabolism, releasing energy and providing carbon for the synthesis of other compounds, such as proteins (Hartmann et al. 2014). Steffens & Rasmussen (2016) point out that adventitious root formation is promoted by an increase in auxin concentration, by increasing sugar transport to the site of root primordia development, thereby ensuring energy supply for growth (Bhatla & Lal 2023). These results are consistent with data from *T₁* trial and confirm the effectiveness of IBA application for promoting root formation in *P. patula* cuttings, as reported in previous studies (Rivera-Rodríguez et al. 2016, Bautista-Ojeda et al. 2022), although rooting percentages vary between studies because of other factors involved.

Biochemical studies show that exogenously applied IBA induces changes in enzyme, protein, and sugar metabolism (Elmongy et al. 2018, de Almeida et al. 2020). Some authors found an increase in soluble sugars in the rooting zone of cuttings of various species after exogenous auxin application (Agulló-Antón et al. 2014, Steffens & Rasmussen 2016, Elmongy et al. 2018). Da Costa et al. (2013) mention that interactions between growth regulators and sugars are an essential part of the sugar sensing and signaling network; stimulation of adventitious rooting by increased IAA coincides with higher sugar availability at the site where root primordia develop (Agulló-Antón et al. 2011).

In this work, etiolation of stock plant for six days combined with immersion in 5000 ppm IBA for 120 s showed an additive effect on rooting capacity of cuttings (64.4 %) after 12 weeks, with a higher number and length of roots, and higher PRS. This result is close to what has been reported for this species, with 73.8 % rooting after 14 weeks, achieved using 18-months-old stock plants with a fertilization routine of 5 g L⁻¹ Osmocote and application of 5000 ppm IBA in liquid solution (Bautista-Ojeda et al. 2022). Therefore, controlling stock plant etiolation and

IBA application at the base of cuttings provides an operationally efficient protocol for cloning *P. patula* cuttings.

Acknowledgments

To the owners of the Multifunctional Forest Reserve “El Manantial” for providing *Pinus patula* seed for stock plant production. To the Secretariat of Science, Humanities, Technology, and Innovation (SECIHTI) for the financial support granted to student Georgina Irasema during her Doctorate program in Forest Sciences at the Colegio de Postgraduados.

References

- Abdel-Mohsen MAA, Rashedy AAH (2023) Stock plant etiolation reduces rooting of sub-terminal olive cuttings by reducing total sugars, IAA, indole/phenol ratio, and IAA/GA ratio. *Acta Physiologiae Plantarum* 45(9): 104. DOI: <https://doi.org/10.1007/s11738-023-03582-z>
- Agulló-Antón MÁ, Ferrández-Ayela A, Fernández-García N, Nicolás C, Albacete A, Pérez-Alfocea F, Sánchez-Bravo J, Pérez-Pérez JM, Acosta M (2014) Early steps of adventitious rooting: Morphology, hormonal profiling and carbohydrate turnover in carnation stem cuttings. *Physiologia Plantarum* 150(3): 446–462. DOI: <https://doi.org/10.1111/ppl.12114>
- Agulló-Antón MÁ, Sánchez-Bravo J, Acosta M, Druege U (2011) Auxins or sugars: what makes the difference in the adventitious rooting of stored carnation cuttings? *Journal of Plant Growth Regulation* 30(1): 100–113. DOI: <https://doi.org/10.1007/s00344-010-9174-8>
- Aloni R. 2013. Role of hormones in controlling vascular differentiation and the mechanism of lateral root initiation. *Planta* 238(5): 819–830. DOI: <https://doi.org/10.1007/s00425-013-1927-8>
- Apine I, Freidenfelds K, Megre D, Dokane K, Kondratovics U. (2013) The effect of stock plant etiolation on rooting and overwinter survival of Deciduous azalea cuttings. *Acta Horticultura* 990: 465–472. DOI: <https://doi.org/10.17660/ActaHortic.2013.990.60>
- Araujo-Viera de Souza JC, Bender AG, Reutemann AG, Perreta MG, Córdoba MS, Tivano JC, Barroso DG, Gariglio NF, Mroginski LA, Vegetti AV. (2021) Influencia del grado de lignificación de los propágulos de jardín y minijardín clonal en el enraizamiento de estacas y miniestacas de *Prosopis alba* Griseb. *Revista FAVE-Ciencias Agrarias* 20(1): 287–304.
- Basuchaudhuri P. (2021) Auxins in rooting of cuttings. *Indian Journal of Plant Sciences* 10: 69–85. <http://www.cibtech.org/jps.htm>
- Bautista-Ojeda GI, Vargas-Hernández JJ, Jiménez-Casas M, López-Peralta MCG. (2022) Manejo de planta y aplicación de IBA en el enraizado de estacas de *Pinus patula*. *Madera y Bosques* 28(1): 1–11.
- Bhatla SC, Lal MA. (2023) *Plant Physiology, Development and Metabolism* (Second Edition). Springer Nature.
- Blommaert KLJ. (1954) Growth and inhibit substances in relation to the rest period of the potato tuber. *Nature* 174: 970–972. DOI: <https://doi.org/10.1038/174970b0>
- da Costa CT, de Almeida MR, Ruedell CM, Schwambach J, Maraschin FS, Fett-Neto AG. (2013) When stress and development go hand in hand: main hormonal controls of adventitious rooting in cuttings. *Frontiers in Plant Science* 4: 133. DOI: <https://doi.org/10.3389/fpls.2013.00133>
- Damodaran S, Strader LC. (2019) Indole 3-butyric acid metabolism and transport in *Arabidopsis thaliana*. *Frontiers in Plant Science* 10: 851. DOI: <https://doi.org/10.3389/fpls.2019.00851>
- de Almeida MR, Schwambach J, Silveira V, Heringer AS, Fett JP, Fett-Neto AG. (2020) Proteomic profiles during adventitious rooting of *Eucalyptus* species relevant to the cellulose industry. *New Forests* 51(2): 213–241. DOI: <https://doi.org/10.1007/s11056-019-09728-7>

- Druege U, Hilo A, Pérez-Pérez JM, Klopotek Y, Acosta M, Shahinnia F, Zerche S, Franken P, Hajirezaei MR. (2019) Molecular and physiological control of adventitious rooting in cuttings: Phytohormone action meets resource allocation. *Annals of Botany* 123(6): 929–949. DOI: <https://doi.org/10.1093/aob/mcy234>
- Druege U, Kadner R. (2008) Response of post-storage carbohydrate levels in pelargonium cuttings to reduced air temperature during rooting and the relationship with leaf senescence and adventitious root formation. *Postharvest Biology and Technology* 47(1): 126–135. DOI: <https://doi.org/10.1016/j.postharvbio.2007.06.008>
- Dumroese RK, Luna T, Landis TD (Eds.) (2009) Nursery manual for native plants: a guide for tribal nurseries. US Department of Agriculture, Forest Service.
- Dvorak, W.S.; Hodge, G.R.; Kietzka, J.E.; Malan, F.; Osorio, L.F.; Stanger, T.K. *Pinus patula*. In Conservation and Testing of Tropical & Subtropical Forest Tree Species; The CAMCORE Cooperative-College of Natural Resources, NCSU: Raleigh, NC, USA, 2000; pp. 149–173.
- Eliyahu A, Duman Z, Sherf S, Genin O, Cinnamon Y, Abu-Abied M, Weinstain R, Dag A, Sadot E. (2020) Vegetative propagation of elite *Eucalyptus* clones as food source for honeybees (*Apis mellifera*); adventitious roots versus callus formation. *Israel Journal of Plant Sciences* 67(1–2): 83–97. DOI: <https://doi.org/10.1163/22238980-20191112>
- Elmongy MS, Cao Y, Zhou H, Xia Y. (2018) Root development enhanced by using indole-3-butyric acid and naphthalene acetic acid and associated biochemical changes of in vitro *Azalea* Microshoots. *Journal of Plant Growth Regulation* 37(3): 813–825. DOI: <https://doi.org/10.1007/s00344-017-9776-5>
- Escamilla-Hernández N, Aldrete A, Vargas-Hernández JJ, López-López MÁ. (2020) Propagación vegetativa de *Pinus patula* Schiede ex Schltdl. et Cham. en diferentes sustratos. *Revista Fitotecnica Mexicana* 43(2): 215. DOI: <https://doi.org/10.35196/rfm.2020.2.215>
- Evert RF. (2006) Esau's plant anatomy. Meristems, cells, and tissues of the plant body: their structure, function, and development (Third Edition). John Wiley & Sons.
- Frick EM, Strader LC. (2018) Roles for IBA-derived auxin in plant development. *Journal of Experimental Botany* 69(2): 169–177. DOI: <https://doi.org/10.1093/jxb/erx298>
- Geiss G, Gutierrez L, Bellini C. (2009) Adventitious root formation: New insights and perspectives. *Annual Plant Reviews: Root Development* 37: 127–156
- Gonin M, Bergougnot V, Nguyen TD, Gantet P, Champion A. (2019) What makes adventitious roots? *Plants* 8(7): 240. DOI: <https://doi.org/10.3390/plants8070240>
- Hamann A. (1998) Adventitious root formation in cuttings of loblolly pine (*Pinus taeda* L.): developmental sequence and effects of maturation. *Trees - Structure and Function* 12: 175–180.
- Harshita GT, Singh G. (2022) The use of auxin and media for rooting of citrus stem cuttings: A review. *The Pharma Innovation Journal* 11(3): 1268–1273. <http://www.thepharmajournal.com>
- Hartmann H, Kester D, Davies F, Geneve R. (2014) Plant propagation: Principles and practices. Prentice Hall. DOI: [https://doi.org/10.1016/0305-1978\(90\)90018-B](https://doi.org/10.1016/0305-1978(90)90018-B)
- Henrique A, Campinhos EN, Ono EO, De Pinho SZ. (2006) Effect of plant growth regulators in the rooting of *Pinus* cuttings. *Brazilian Archives of Biology and Technology* 49(2): 189–196. DOI: <https://doi.org/10.1590/S1516-89132006000300002>
- Husen A. (2008) Stock-plant etiolation causes drifts in total soluble sugars and anthraquinones and promotes adventitious root formation in teak (*Tectona grandis* L. f.) coppice shoots. *Plant Growth Regulation* 54(1): 13–21. DOI: <https://doi.org/10.1007/s10725-007-9222-y>
- Kanmegne G, Mbouobda HD, Fotso Mbakop CN, Omokolo DN. (2017) Influence of stockplant fertilization on tissue concentrations of nitrogen, carbohydrates and amino acids and rooting of leafy stem cuttings of *Cola anomala* K. Schum (*Malvaceae*). *New Forests* 48(1): 17–31. DOI: <https://doi.org/10.1007/s11056-016-9553-5>
- Korasick DA, Enders TA, Strader LC. (2013) Auxin biosynthesis and storage forms. *Journal of Experimental Botany* 64(9): 2541–2555. DOI: <https://doi.org/10.1093/jxb/ert080>
- Kreiser M, Giblin C, Murphy R, Fiesel P, Braun L, Johnson G, Wyse D, Cohen JD. 2016. Conversion of Indole-3-Butyric Acid to Indole-3-Acetic Acid in shoot tissue of Hazelnut (*Corylus*) and Elm (*Ulmus*). *Journal of Plant Growth Regulation* 35(3): 710–721. DOI: <https://doi.org/10.1007/s00344-016-9574-5>
- Legué V, Rigal A, Bhalerao RP. (2014) Adventitious root formation in tree species: Involvement of transcription factors. *Physiologia Plantarum* 151(2): 192–198. DOI: <https://doi.org/10.1111/ppl.12197>
- Lira G DE. (2020) Guía Básica de Pinos Mexicanos. Boskia: México.
- Loewus FA. (1952) Improvement in anthrone method for determination of carbohydrates. *Analytical Chemistry* 24(1): 219.
- Ludwig-Müller J. (2000) Indole-3-butyric acid in plant growth and development. *Plant Growth Regulation* 32: 219–230.
- Majada J, Martínez-Alonso C, Feito I, Kidelman A, Aranda I, Alía R. (2011) Mini-cuttings: An effective technique for the propagation of *Pinus pinaster* Ait. *New Forests* 41(3): 399–412. DOI: <https://doi.org/10.1007/s11056-010-9232-x>
- Pacholczak A, Jędrzejuk A, Sobczak M. (2017) Shading and natural rooting bio-stimulator enhance potential for vegetative propagation of dogwood plants (*Cornus alba* L.) via stem cuttings. *South African Journal of Botany* 109: 34–41. DOI: <https://doi.org/10.1016/j.sajb.2016.12.009>
- Pacholczak A, Szydio W, Łukaszewska A. (2005) The effect of etiolation and shading of stock plants on rhizogenesis in stem cuttings of *Cotinus coggygria*. *Acta Physiologiae Plantarum* 27(4): 417–428. DOI: <https://doi.org/10.1007/s11738-005-0046-y>
- Pacurar DI, Perrone I, Bellini C. (2014) Auxin is a central player in the hormone cross-talks that control adventitious rooting. *Physiologia Plantarum* 151(1): 83–96. DOI: <https://doi.org/10.1111/ppl.12171>
- Pan X, Welti R, Wang X. (2010) Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. *Nature Protocols* 5(6): 986–992. DOI: <https://doi.org/10.1038/nprot.2010.37>
- Park Y S. (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. *Annals of Forest Science*, 59(5–6): 651–656. DOI: <https://doi.org/10.1051/forest:2002051>
- Pizarro A, Díaz-Sala C. (2020) Effect of polar auxin transport and gibberellins on xylem formation in pine cuttings under adventitious rooting conditions. *Israel Journal of Plant Sciences* 67(1–2): 27–39. DOI: <https://doi.org/10.1163/22238980-20191120>
- Rioy J, Foxa H, Attiasa R, Shklar G, Farkash-Haim L, Sitbon R, Moshe Y, Abu-Abied M, Sadot E, David-Schwartz R. (2020) Improved method for vegetative propagation of mature *Pinus halepensis* and its hybrids by cuttings. *Israel Journal of Plant Sciences* 67(1–2): 5–15. DOI: <https://doi.org/10.1163/22238980-20191118>
- Rivera-Rodríguez M, Vargas-Hernández JJ, López-Upton J, Villegas-Monter Á, Jiménez-Casas M. (2016) Enraizamiento de estacas de *Pinus patula*. *Revista Fitotecnica Mexicana* 39(4): 385–392. DOI: <https://doi.org/10.35196/rfm.2020.2.215>
- Statistical Analysis System. (2013) SAS 9.4 [Computer software]. Institute Inc. NC USA. 5121p
- Steffens B, Rasmussen A. (2016) The physiology and molecular biology of adventitious root formation. *Plant Physiology* 170(2): 603–617. DOI: <https://doi.org/10.1104/pp.15.01360>
- Strader LC, Bartel B. (2011) Transport and metabolism of the endogenous auxin precursor indole-3-butyric acid. *Molecular Plant* 4(3): 477–486. DOI: <https://doi.org/10.1093/mp/ssr006>
- Wei H, Zhao H, Chen X, He X. (2020) Secondary metabolites, carbohydrate accumulation, and nutrient uptake in *Aralia elata* (Miq.) Seem seedlings exposed to shoot cutting and different LED spectra. *Acta Physiologiae Plantarum* 42(11):162. DOI: <https://doi.org/10.1007/s11738-020-03149-2>
- White, T. L., Adams, W. T., & Neale, D. B. (Eds.). (2007). *Forest genetics*. Cabi.
- Wright, J. A., Osorio, L. F., & Dvorak, W. S. (1996). Realised and predicted genetic gain in the *Pinus patula* breeding program of Smurfit Carton de Colombia. *South African Forestry Journal*, 175(1), 19–22.
- Zavaleta-Mancera HA, Engleman EM. (1994) Anatomy of the ovule and seed of *Manilkara zapota* (L.) Van Royen (*Sapotaceae*). *Phytomorphology* 44: 169–175.
- Zerche S, Haensch KT, Druege U, Hajirezaei MR. (2016) Nitrogen remobilisation facilitates adventitious root formation on reversible dark-induced carbohydrate depletion in *Petunia* hybrida. *BMC Plant Biology* 16(1): 219. DOI: <https://doi.org/10.1186/s12870-016-0901-6>

Zhou Y, Wei K, Hao X, Wang L, Li N, Zhang W, Tang S, Li D, Zhang W. (2025) Differential rooting efficacy of growth regulators in *Camellia sinensis* Cuttings: A physiological and biochemical analysis. Horticulturae 11(3): 289. DOI: <https://doi.org/10.3390/horticulturaT11030289>