

## Article

# Effects of the Most Appropriate Proportion of Phytohormones on Tree-Ring Growth in Clones of Hybrid Larch

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**Abstract:** The increase in the atmospheric carbon dioxide concentration promotes its accumulation in trees by regulating the synthesis and transportation genes for endogenous hormones, such as IAA and GA, which are key factors in regulating various life activities, including growth rings. To explore the impact of changes in endogenous hormone levels such as IAA and GA on the growth of tree rings, and to provide a basis for improving the management of hybrid larch clonal forests, we investigated the effects of exogenous indole-3-acetic acid (IAA), gibberellic acid 3 (GA<sub>3</sub>), and their combination on tree-ring growth in hybrid larch. IAA, GA<sub>3</sub>, and a combination treatment were sprayed on the leaves of one clone of a hybrid larch seedling every three days. Small blocks were collected at the base stems for sequential anatomical observations. The phytohormone type, instead of the concentration, had a more significant effect on wood formation. Specifically, IAA treatment at 300 mg L<sup>-1</sup> significantly increased latewood (LW) layers until 90 days after treatment (DAT). The 500 mg L<sup>-1</sup> treatment significantly increased the wall radial thickness (WRT) of latewood (LW) cells. GA<sub>3</sub> treatment at 100 mg L<sup>-1</sup> significantly decreased the layers and width of total wood (TW), LW, and earlywood (EW). The 300 mg L<sup>-1</sup> treatment significantly increased the WRT of EW. The IAA 100 mg L<sup>-1</sup> + GA<sub>3</sub> 100 mg L<sup>-1</sup> combination treatment significantly increased the layers and width of TW and LW by inducing cambium activity and increasing the rate of wood cell development. The WRT and lumen radial diameter (LRD) of EW or LW in this treatment were similar to those observed with the corresponding single phytohormone treatment. These results indicate that combination treatment at 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> was a better way to promote tree-ring growth. Our study suggests that changes in phytohormone levels and ratios are important factors that affect tree-ring formation. Hormone levels and ratios should be regarded as important indicators to guide the improvement of management practices in hybrid larch clonal plantations.

**Keywords:** larch; hybrid; wood formation; earlywood; latewood; auxin; gibberellin



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## 1. Introduction

The increasing CO<sub>2</sub> (eCO<sub>2</sub>) in the atmosphere affects stomatal closure through the independent signal transduction pathway [1] or mediation by other phytohormone signaling transduction pathways [2]. Increasing CO<sub>2</sub> can promote the accumulation of indole-3-acetic acid (IAA) and gibberellins (GAs), which regulate plant growth and development by promoting the expression of the auxin transporting gene *LAX* [3], IAA biosynthetic gene *FZY* [4], and GA biosynthetic gene *GGPPS* [5]. Specifically, eCO<sub>2</sub> was shown to increase IAA content to 3 µg g<sup>-1</sup> in *Ginkgo biloba* L. [6], which was higher than that in the control of 2 µg g<sup>-1</sup>. In *Arabidopsis thaliana* [7], the concentration of IAA and gibberellin A3 (GA<sub>3</sub>) increased from 31.27 ± 2.87 ng g<sup>-1</sup> fresh weight (FW) and 211.05 ± 17.73 ng g<sup>-1</sup> FW to 35.56 ± 2.97 and 327.96 ± 30.81 ng g<sup>-1</sup> FW, respectively. In addition, in response to biotic and abiotic stresses, eCO<sub>2</sub> promotes the accumulation of corresponding phytohormones

such as ethylene [8], jasmonic acid, and salicylic acid [5,9–11]. Furthermore, it has been proven that IAA and GA are two of the most important phytohormones regulating tree-ring growth [12,13].

The most common auxin in most plants is indole-3-acetic acid (IAA). It affects many plant activities, including vascular development, cambium activity [14,15], and tree-ring growth [16,17]. IAA is the most studied plant hormone [18], and a clear pattern of its regulation of tree-ring growth has been established. In the early part of the growing season, the supply of IAA from young leaves and leaf primordia to the cambium is necessary for the production of xylem cells [19]. When the endogenous IAA level and the cambial growth rate reach the maximum, the xylem experiences its highest period of activity [20]. Endogenous IAA levels, after peaking when cambial and xylem differentiation are most active, decrease before latewood formation and then change only slightly until the end of the growing season and through cambial dormancy [20,21]. In gymnosperms, latewood formation is linked to a steeper IAA distribution gradient in the cambial and differentiating cells. Many experiments have also confirmed that IAA can induce cambium proliferation and xylem production. This pattern has been linked to a decrease in cell division and differentiation [20].

Previous studies have shown that gibberellins (gibberellic acids, GAs) also affect tree-ring growth [22–26]. GAs are mainly distributed in the differentiating xylem cells in the stem of trees [27]. Studies have shown that GAs, like IAA, also affect the cambium division in the cambial zone but do not affect xylogenesis, resulting in the apparent loss of an easily distinguished cambial meristem. Possibly the auxin deficiency in these stems results in de-differentiation and loss of meristem identity of the newly formed cells. GA<sub>3</sub> application treatment also leads to wider xylem development and higher lignin content than controls [28,29]. These results indicate that GAs may mainly be involved in xylem differentiation and secondary cell wall (SCW) deposition in tree-ring formation. Furthermore, the distribution pattern has shown that IAA and GAs overlap in developing xylem cells during tree-ring growth [18]. In angiosperms, IAA with GA<sub>3</sub> promotes more wood formation than either individually [30].

In conifers, few studies have reported the effects of auxin and GAs together on tree-ring growth (earlywood and latewood). Only Wang has reported that GA<sub>4/7</sub> and IAA together, applied to the decapitated and defoliated current-year shoots of *Pinus sylvestris* increased shoot elongation, xylem, and phloem production, and IAA concentration in the early part of the growing season [31]. However, the effects of combination treatment with auxin and GAs on tree-ring growth and latewood formation are not completely clear yet in conifers. Larch is widely distributed in the northeast of Eurasia and is one of the four major afforestation species in China. Its wood can be used for construction and paper making. In this study, in order to investigate the effects of phytohormone levels such as IAA and GA<sub>3</sub> together on tree-ring growth, especially in respect of latewood formation, we used a clone of 3-year-old hybrid larch as the material and conducted experimental treatments with exogenous IAA and GA<sub>3</sub> to clarify the changes in tree-ring growth under single or combined hormone treatments. This study will provide a theoretical basis and application measures for improving the management model of larch plantations from the aspect of phytohormones.

## 2. Materials and Methods

### 2.1. Plant Material

Three-year-old hybrid larch trees (*Larix Kaempferi* 302 × *Larix olgensis* 23 8–14) were grown in the field of Da Gu Jia Town, Liaoning Province, China, and were used in the present study. The growth medium was a sterile mixture of peat:perlite (2.5:1) in an adhesively bonded fabric planting bag of 20 cm × 25 cm (height × diameter).

## 2.2. Experimental Design and Phytohormone Treatments

### 2.2.1. Experimental Design

Ten treatments were performed: IAA ( $100 \text{ mg L}^{-1}$ ,  $300 \text{ mg L}^{-1}$ , and  $500 \text{ mg L}^{-1}$ ), IAA + GA<sub>3</sub> ( $100 \text{ mg L}^{-1}$  +  $100 \text{ mg L}^{-1}$ ,  $300 \text{ mg L}^{-1}$  +  $100 \text{ mg L}^{-1}$ , and  $500 \text{ mg L}^{-1}$  +  $100 \text{ mg L}^{-1}$ ), GA<sub>3</sub> ( $100 \text{ mg L}^{-1}$ ,  $300 \text{ mg L}^{-1}$ , and  $500 \text{ mg L}^{-1}$ ), and distilled water as the control (CK). One hundred and twenty seedlings in total were divided into three replicates with forty seedlings per replicate. Four seedlings per treatment per replicate were treated, and all ten treatments were ordered randomly per replicate. All treated seedlings were kept in Da Gu Jia Town, Liaoning Province, China, with the same growing conditions of natural photoperiod and temperature.

### 2.2.2. Phytohormone Treatment

Indole-3-acetic acid (Genview, Beijing, China) and gibberellin A3 (Shanghai Ryon Biological Technology Co., Ltd., Shanghai, China) were dissolved in a small amount of 99% ethanol, then dissolved in distilled water with 0.05% Tween-20 (Sigma-Aldrich, Shanghai, China) to the corresponding concentration for every treatment. Seedlings were sprayed every 3 days with 4 mL of the mixture from 20 May to 21 September 2019. During the spraying treatments, the leaves of each seedling were covered with hormone solution to form droplets to ensure that each seedling could absorb the hormone solution in every treatment.

### 2.3. Collection of Samples

One block of about  $0.3 \text{ cm}^3$  was collected and fixed in 4% paraformaldehyde (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) from the base stem (2 cm above medium) of one seedling per treatment per replicate from 20 June (30 days after treatment: 30DAT) to 21 September, with an interval of 30 days. Additionally, one block was collected from each of three seedlings (i.e., three in total), on 20 May (day 0) to confirm the stage of tree-ring growth at the beginning of the experiment.

### 2.4. Anatomical Observations

For anatomical observations, the blocks were dehydrated through an ethanol series (ethanol:distilled water) with 75% (v/v), 90% (v/v), and pure ethanol, and then embedded into Spurr's resin (Low Viscosity Embedding Media Spurr's kit, Garfield Ave., West Chester, PA, USA) with ethanol in a series of 25% (v/v), 50% (v/v), and 75% (v/v). Each concentration of ethanol and resin was applied for two hours. Then the blocks were treated with pure resin three times for twelve hours at a time. Transverse sections about  $4 \mu\text{m}$  thick were cut using a Leica microtome (LECICA EM UC7, Wetzlar, Germany), spread on a slide with fine tweezers, stained with 0.25% (w/v) toluidine blue O (Sigma, Shanghai, China) and observed under a microscope (Carl Zeiss Microimaging GmbH37081, Oberkochen, Germany).

### 2.5. Anatomical Measurements of Cell Morphology and Statistical Analysis

From the growth ring boundary formed in the last year toward the cambial zone, cells of three radial lines, selected randomly from the middle and both sides of one sample, for nine radial lines from three samples in total, were analyzed under every treatment. The layers of total wood (TW), earlywood (EW), latewood (LW), and cambium were collected on 20 May (0 days after treatment: 0 DAT), 27 June (30 DAT), 23 July (60 DAT), 24 August (90 DAT), and 21 September (120 DAT). The transverse sections of samples collected at 120 DAT were further measured and analyzed, including the width of the newly formed xylem (total wood), EW, and LW; the wall radial thickness (WRT); and the lumen radial diameter (LRD) of xylem cells using image analysis software (Image J; National Institutes of Health, Bethesda, MD, USA). Then, the ratio of LW to total wood (TW) was calculated. Each statistical analysis was performed using IBM SPSS Statistics 19.0 (IBM, Armonk, NY, USA). The data were analyzed using Duncan's multiple-range test ( $p < 0.05$ ) in triplicate. The wood cell development pattern was analyzed using the Gompertz function [32].

### 3. Results

#### 3.1. ANOVA Analysis Revealed That IAA + GA<sub>3</sub> Treatment Was a More Efficient Way to Promote Tree-Ring Growth

ANOVA analysis showed that the type of phytohormone significantly affected growth and tree-ring growth in hybrid larch seedlings. Indole-3-acetic acid (IAA) treatments only significantly decreased the earlywood layers (EWLs). Gibberellin A3 (GA<sub>3</sub>) treatments significantly increased the increment of height (IH) but significantly decreased the total wood layers (TWLs), EWLs, and latewood layers (LWLs). Combination treatments significantly increased the increment of base stem diameter (IBD), TWLs, LWLs, and cambium layers (CLs), but the survival rate (SR) was significantly decreased. The concentration of phytohormones significantly affected all of the phenotypes except TWL and LWL. A low concentration (100 mg L<sup>-1</sup> in IAA or GA<sub>3</sub> treatment and 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> in combination treatment) significantly increased IH and IBD, but significantly decreased EWLs. A medium concentration (300 mg L<sup>-1</sup> in IAA or GA<sub>3</sub> treatment and 300 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> in combination treatment) significantly increased IH and CLs but significantly decreased EWLs. A high concentration (500 mg L<sup>-1</sup> in IAA or GA<sub>3</sub> treatment and 500 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> in combination treatment) significantly increased CLs but significantly decreased the SR (Table 1).

**Table 1.** ANOVA results for phenotypes under different phytohormone treatments.

|     |         | Phytohormone Type |        |                 |         | Concentration |         |        |        |
|-----|---------|-------------------|--------|-----------------|---------|---------------|---------|--------|--------|
|     |         | CK                | IAA    | GA <sub>3</sub> | IG      | CK            | LCTs    | MCTs   | HCTs   |
| IH  | Average | 5.81              | 6.14   | 11.00 *         | 7.09    | 5.81          | 10.41 * | 7.28 * | 6.05   |
|     | F       |                   |        |                 |         |               |         |        |        |
| IBD | Average | 0.19              | 0.22   | 0.19            | 0.30 *  | 0.19          | 0.28 *  | 0.20   | 0.22   |
|     | F       |                   |        |                 |         |               |         |        |        |
| SR  | Average | 1.00              | 1.00   | 0.92            | 0.83*   | 1.00          | 1.00    | 0.92   | 0.83 * |
|     | F       |                   |        |                 |         |               |         |        |        |
| TWL | Average | 10.89             | 10.73  | 8.49 *          | 13.03 * | 10.89         | 10.65   | 11.03  | 10.34  |
|     | F       |                   |        |                 |         |               |         |        |        |
| EWL | Average | 6.33              | 5.48 * | 5.28 *          | 6.13    | 6.33          | 5.16 *  | 5.54 * | 6.24   |
|     | F       |                   |        |                 |         |               |         |        |        |
| LWL | Average | 4.56              | 5.25   | 3.05 *          | 6.90*   | 4.56          | 5.49    | 5.33   | 4.10   |
|     | F       |                   |        |                 |         |               |         |        |        |
| CL  | Average | 3.47              | 3.61   | 3.22            | 4.54 *  | 3.47          | 3.51    | 3.84 * | 3.99 * |
|     | F       |                   |        |                 |         |               |         |        |        |
|     | P       |                   |        |                 |         |               |         |        |        |

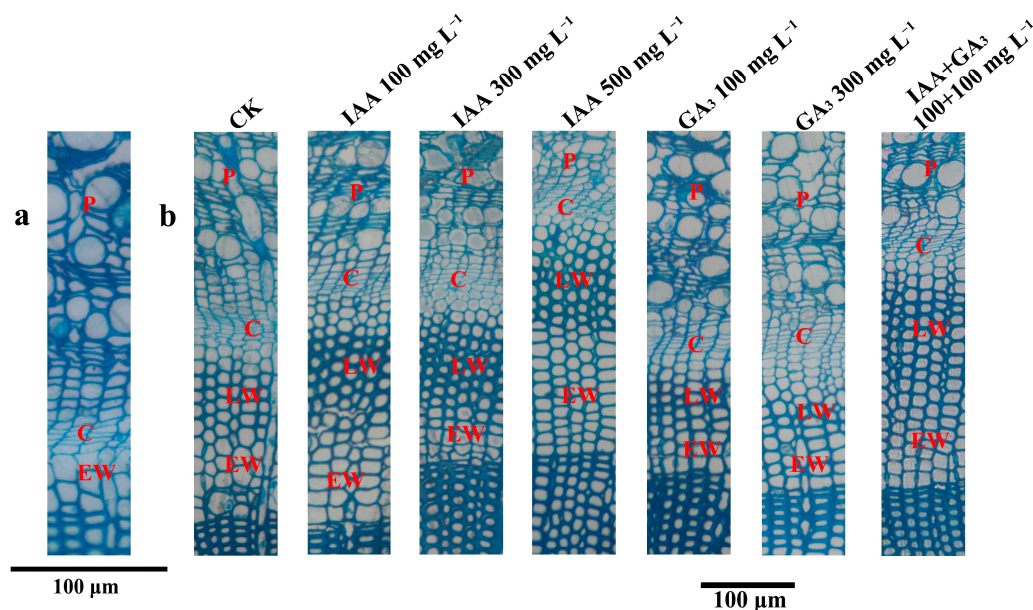
IAA: IAA treatments; GA<sub>3</sub>: GA<sub>3</sub> treatments; IG: IAA + GA<sub>3</sub> combination treatments; LCTs: low-concentration treatments including IAA 100 mg L<sup>-1</sup>, GA<sub>3</sub> 100 mg L<sup>-1</sup>, and IAA 100 mg L<sup>-1</sup> + GA<sub>3</sub> 100 mg L<sup>-1</sup>; MCTs: medium-concentration treatments including IAA 300 mg L<sup>-1</sup>, GA<sub>3</sub> 300 mg L<sup>-1</sup>, and IAA 300 mg L<sup>-1</sup> + GA<sub>3</sub> 100 mg L<sup>-1</sup>; HCTs: high-concentration treatments including IAA 500 mg L<sup>-1</sup>, GA<sub>3</sub> 500 mg L<sup>-1</sup>, and IAA 500 mg L<sup>-1</sup> + GA<sub>3</sub> 100 mg L<sup>-1</sup>. IH: increment of height. IBD: increment of base stem diameter. SR: survival rate. TWLs: total wood layers. EWLs: earlywood layers. LWLs: latewood layers. CLs: cambium layers. \*:  $p < 0.05$ .

#### 3.2. Tree-Ring Growth under Different Phytohormone Treatments

##### 3.2.1. Development of Total Wood Layers

About three to four layers of newly formed xylem cells (total wood layers) and about five layers of cambium cells were detected at 0 days after treatment (DAT) (Figure 1a). At 30 DAT, IAA treatments with 100 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> significantly decreased the TWLs. The TWLs were also significantly decreased at GA<sub>3</sub> treatment with 100 mg L<sup>-1</sup>. Combination treatment at 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> significantly increased the TWLs.

At 60 DAT, both IAA treatments and GA<sub>3</sub> treatments at 100 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> significantly decreased the TWLs. All the combination treatments (100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup>, 300 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup>, and 500 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup>) significantly increased the TWLs. At 90 DAT, IAA treatment at 300 mg L<sup>-1</sup> significantly increased the TWLs. GA<sub>3</sub> treatments at 100 mg L<sup>-1</sup> and 300 mg L<sup>-1</sup> significantly decreased the TWLs. All three combination treatments significantly increased the TWLs. At 120 DAT, IAA treatments had no effect on the TWLs. GA<sub>3</sub> treatment at 100 mg L<sup>-1</sup> and 300 mg L<sup>-1</sup> significantly decreased the TWLs. The combination treatment at 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> significantly increased the TWLs.



**Figure 1.** Transverse sections of hybrid larch at 0 DAT and 120 DAT. (a) Transverse section at 0 DAT. (b) Transverse sections at 120 DAT. P: phloem; C: cambium; EW: earlywood; LW: latewood.

### 3.2.2. Development of Earlywood Layers

For EWLs, at 30 DAT, IAA treatments at 100 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> significantly decreased the EWLs. GA<sub>3</sub> treatment at 100 mg L<sup>-1</sup> significantly decreased the EWLs. Similarly, the EWLs were significantly decreased by combination treatment with 300 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup>. At 60 DAT, IAA treatments had no significant effect on the EWLs. The EWLs were significantly decreased by GA<sub>3</sub> treatment at 100 mg L<sup>-1</sup> but significantly increased at 500 mg L<sup>-1</sup>. The combination treatment at 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> significantly increased the EWLs. At 90 DAT, IAA treatment at 100 mg L<sup>-1</sup> significantly decreased the EWLs. All three GA<sub>3</sub> treatments significantly decreased the EWLs. The combination treatments had no significant effect on EWLs. At 120 DAT, IAA treatment at 100 mg L<sup>-1</sup> significantly decreased the EWLs. GA<sub>3</sub> treatments at 100 mg L<sup>-1</sup> and 300 mg L<sup>-1</sup> significantly decreased the EWLs. There was no significant difference between the combination treatment at 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> and CK.

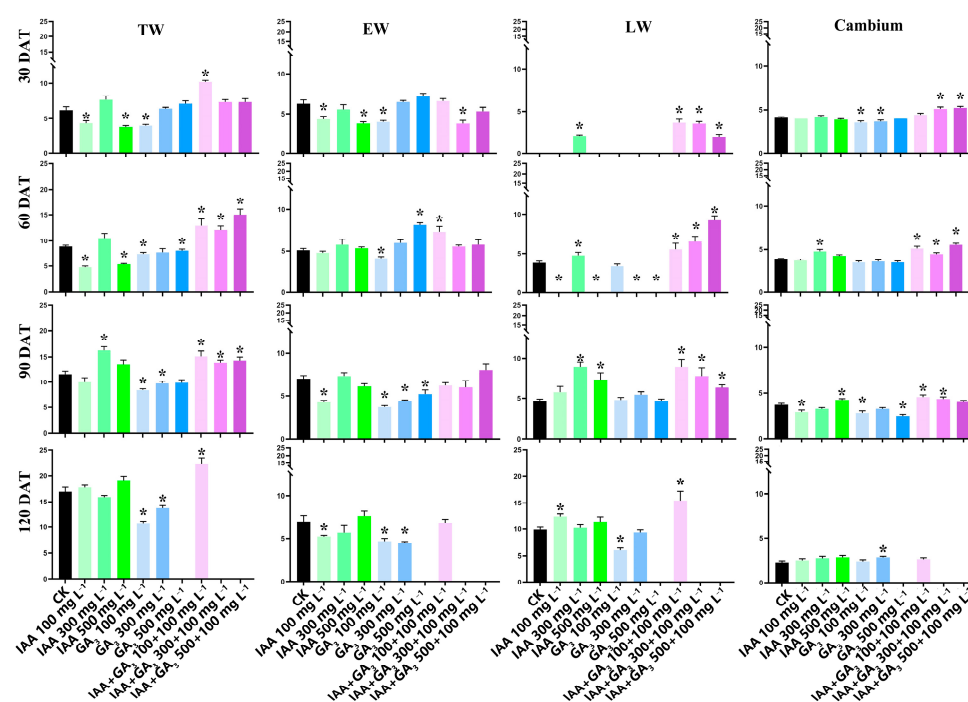
### 3.2.3. Development of Latewood Layers

For LWLs, at 30 DAT, IAA treatment at 300 mg L<sup>-1</sup> significantly increased LW layers (LWLs). No LW was found in GA<sub>3</sub> treatments. The LWLs were significantly increased by all three combination treatments. At 60 DAT, IAA treatment at 300 mg L<sup>-1</sup> significantly increased the LWLs, while no LW was found in the other two IAA treatments. There was no significant difference between GA<sub>3</sub> treatment at 100 mg L<sup>-1</sup> and CK. All three combination treatments significantly increased the LWLs. At 90 DAT, IAA treatments at 300 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> significantly increased the LWLs. GA<sub>3</sub> treatments had no effect on LWLs. All three combination treatments significantly increased LWLs. At 120 DAT, IAA treatment at

100 mg L<sup>-1</sup> significantly increased the LWLs. GA<sub>3</sub> treatment at 100 mg L<sup>-1</sup> significantly decreased the LWLs. Combination treatment at 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> significantly increased the LWLs.

### 3.2.4. Development of Cambium Layers

At 30 DAT, the cambium layers (CLs) were similar at all three concentrations of IAA treatment. The CLs were significantly decreased by GA<sub>3</sub> treatments at 100 mg L<sup>-1</sup> and 300 mg L<sup>-1</sup>. Combination treatments with 300 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> significantly increased the CLs. At 60 DAT, IAA treatment at 300 mg L<sup>-1</sup> significantly increased the CLs. There was no significant difference between GA<sub>3</sub> treatments and CK. All three combination treatments significantly increased the CLs. At 90 DAT, IAA treatments at a low concentration (100 mg L<sup>-1</sup>) significantly decreased the CLs, while a high concentration (500 mg L<sup>-1</sup>) significantly increased the CLs. GA<sub>3</sub> treatments at 100 mg L<sup>-1</sup> and 500 mg L<sup>-1</sup> significantly decreased the CLs. Combination treatments at 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> and 300 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> significantly increased the CLs. At 120 DAT, only 2–3 cambium layers were detected in all of the treatments and CK. Combination treatments and IAA treatments had no effect on the CLs, but GA<sub>3</sub> treatment at 300 mg L<sup>-1</sup> significantly increased the CLs (Figures 1b and 2).



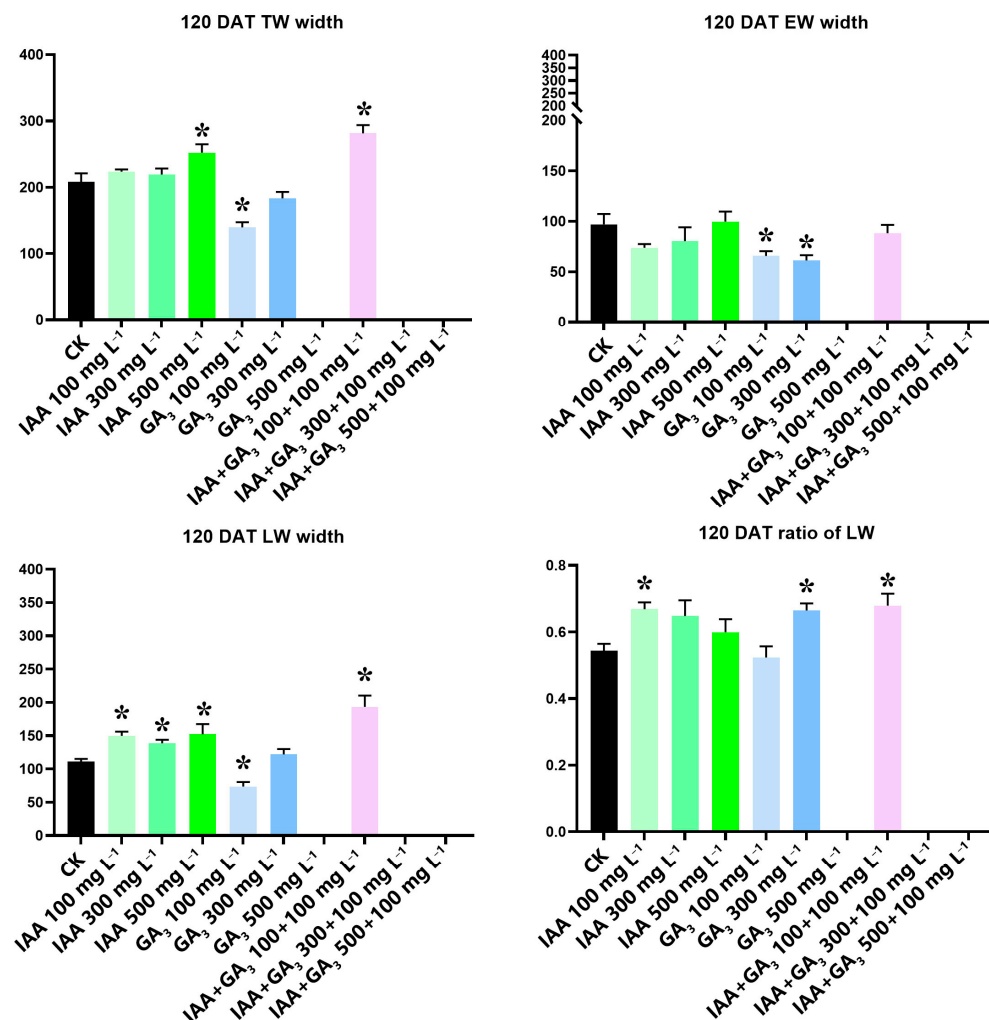
**Figure 2.** Calculation of total wood layers, earlywood layers, latewood layers, and cambium layers during the experiment. The T-test was used, and the least significant range analysis at 5% significance is shown with \* at the same stage for the control and treatments. All data were calculated in triplicate.

### 3.3. Calculation of Wood Cell Morphology under Different Phytohormone Treatments

#### 3.3.1. Calculation of Newly Formed Wood Width, including Latewood and Earlywood

We measured the total wood width (TWW), earlywood width (EWW), and latewood width (LWW) in 2019 for the present study at 120 DAT. Then, the ratio of LWW to TWW was calculated. The IAA treatment at 500 mg L<sup>-1</sup> significantly increased the TWW. GA<sub>3</sub> treatment at 100 mg L<sup>-1</sup> significantly decreased TWW. Combination treatment at 100 mg L<sup>-1</sup> + 100 mg L<sup>-1</sup> significantly increased TWW. IAA treatments had no significant effect on earlywood width (EWW). GA<sub>3</sub> treatments at 100 mg L<sup>-1</sup> and 300 mg L<sup>-1</sup> significantly decreased EWW. Combination treatment had no significant effect on EWW. All three IAA treatments significantly increased LWW. GA<sub>3</sub> treatment at 100 mg L<sup>-1</sup> significantly

decreased LWW. Combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  significantly increased LWW. The ratio of LWW to TWW was significantly increased by IAA treatment at  $100 \text{ mg L}^{-1}$ ,  $\text{GA}_3$  treatment at  $300 \text{ mg L}^{-1}$ , and combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  (Figure 3).



**Figure 3.** Measurement and calculation of newly formed wood width, earlywood width, and the ratio of latewood width to total wood width at 120 DAT. The T-test was used, and the least significant range analysis at 5% significance is shown at the same stage with \* for the control and treatments. All data were calculated in triplicate.

### 3.3.2. Measurement of Wall Radial Thickness and Lumen Radial Diameter in Latewood and Earlywood

The wall radial thickness (WRT) and lumen radial diameter (LRD) of EW and LW were measured, respectively, and the ratio of WRT to LRD was calculated (Table 2). Only  $\text{GA}_3$  treatment at  $300 \text{ mg L}^{-1}$  significantly increased the WRT of EW. The three IAA treatments and the combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  had no significant effect on the WRT of EW. The LRD of EW was significantly decreased by IAA treatments at  $100 \text{ mg L}^{-1}$  and  $500 \text{ mg L}^{-1}$ .  $\text{GA}_3$  treatment at  $100 \text{ mg L}^{-1}$  significantly decreased the LRD of EW but significantly increased it at  $300 \text{ mg L}^{-1}$ . Combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  significantly decreased the LRD of EW. The ratio of WRT to LRD in EW was not significantly affected in any of the treatments. The WRT of LW was significantly decreased by IAA treatments at low and middle concentrations ( $100 \text{ mg L}^{-1}$  and  $300 \text{ mg L}^{-1}$ ) but was significantly increased by a high concentration ( $500 \text{ mg L}^{-1}$ ).  $\text{GA}_3$  treatments at  $100 \text{ mg L}^{-1}$  and  $300 \text{ mg L}^{-1}$  significantly decreased the WRT of LW. Similarly, combination treatment

at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  significantly decreased the WRT of LW. The LRD of LW was significantly decreased by IAA treatments and  $\text{GA}_3$  treatments at low and middle concentrations ( $100 \text{ mg L}^{-1}$  and  $300 \text{ mg L}^{-1}$ ). Combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  significantly decreased the LRD of LW. The ratio of WRT to LRD in LW was significantly increased under combination treatment,  $\text{GA}_3$  treatments, and IAA treatments (Table 2).

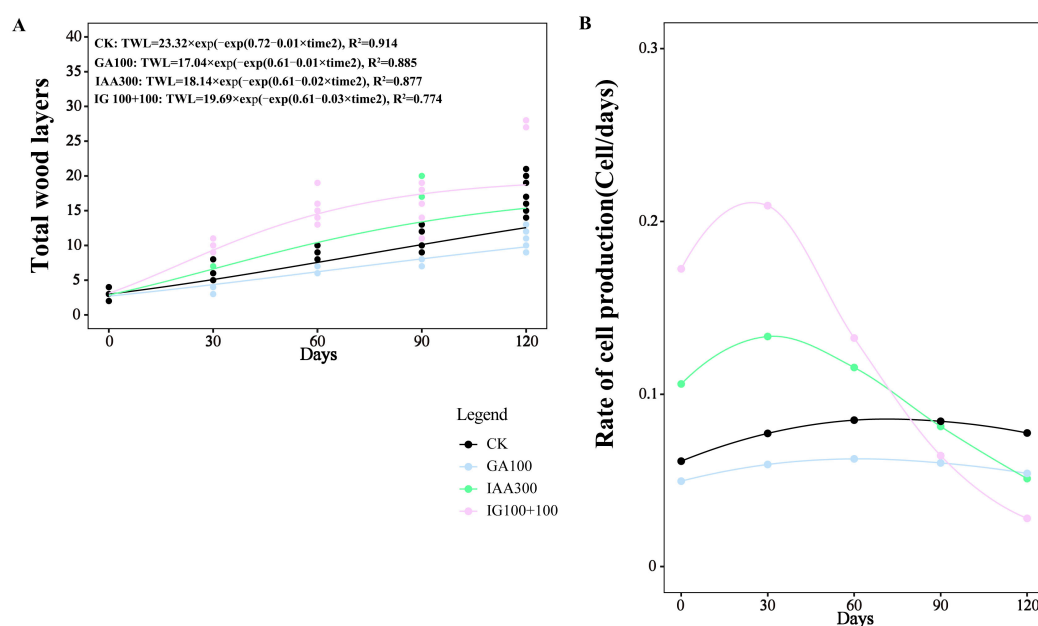
**Table 2.** Measurement of wood cell morphology at 120 DAT.

|       | WRTEW ( $\mu\text{m}$ ) | LRDEW ( $\mu\text{m}$ ) | RWLEW           | WRTLW ( $\mu\text{m}$ ) | LRDLW ( $\mu\text{m}$ ) | RWLLW             |
|-------|-------------------------|-------------------------|-----------------|-------------------------|-------------------------|-------------------|
| CK    | $1.88 \pm 0.13$         | $12.26 \pm 0.35$        | $0.31 \pm 0.02$ | $6.27 \pm 0.37$         | $15.11 \pm 0.84$        | $0.85 \pm 0.04$   |
| I100  | $1.73 \pm 0.07$         | $10.68 \pm 0.31^*$      | $0.34 \pm 0.02$ | $4.29 \pm 0.14^*$       | $8.30 \pm 0.25^*$       | $1.06 \pm 0.03^*$ |
| I300  | $1.74 \pm 0.07$         | $13.18 \pm 0.52$        | $0.28 \pm 0.01$ | $4.56 \pm 0.21^*$       | $8.67 \pm 0.24^*$       | $1.14 \pm 0.08^*$ |
| I500  | $1.83 \pm 0.07$         | $10.93 \pm 0.31^*$      | $0.34 \pm 0.01$ | $7.27 \pm 0.33^*$       | $16.17 \pm 1.02$        | $0.99 \pm 0.04^*$ |
| G100  | $1.81 \pm 0.05$         | $10.88 \pm 0.31^*$      | $0.34 \pm 0.01$ | $4.38 \pm 0.19^*$       | $6.59 \pm 0.23^*$       | $1.41 \pm 0.09^*$ |
| G300  | $2.42 \pm 0.23^*$       | $17.21 \pm 1.74^*$      | $0.30 \pm 0.01$ | $4.10 \pm 0.10^*$       | $6.45 \pm 0.22^*$       | $1.32 \pm 0.05^*$ |
| IG100 | $1.63 \pm 0.09$         | $10.81 \pm 0.38^*$      | $0.31 \pm 0.02$ | $4.34 \pm 0.11^*$       | $7.63 \pm 0.32^*$       | $1.24 \pm 0.06^*$ |

I100: IAA  $100 \text{ mg L}^{-1}$  treatment. I300: IAA  $300 \text{ mg L}^{-1}$  treatment. I500: IAA  $500 \text{ mg L}^{-1}$  treatment. G100:  $\text{GA}_3$   $100 \text{ mg L}^{-1}$  treatment. G300:  $\text{GA}_3$   $300 \text{ mg L}^{-1}$  treatment. IG100:  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  IAA +  $\text{GA}_3$  treatment. WRTEW: wall radial thickness of earlywood. LRDEW: lumen radial diameter of earlywood. RWLEW: ratio of WRTEW to LRDEW. WRTLW: wall radial thickness of latewood. LRDLW: lumen radial diameter of latewood. RWLLW: ratio of WRTLW to LRDLW. The T-test was used, and the least significant range analysis at 5% significance is shown by \* at the same stage for CK and treatments. All data were calculated in triplicate. Data are shown as means  $\pm$  SEM.

### 3.4. The Pattern of Tree-Ring Growth

To obtain the tree-ring growth pattern, we used the Gompertz function to model the increase in the wood cell number (TWLs) for the three most representative treatments, including the treatment that most significantly promoted tree-ring growth ( $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  combination treatment), the treatment significantly that most inhibited the tree-ring growth ( $100 \text{ mg L}^{-1}$   $\text{GA}_3$  treatment), the treatment that had no significant effect on tree-ring growth ( $300 \text{ mg L}^{-1}$  IAA treatment), and CK in the present study. The dynamics of TWLs fitted the Gompertz function, with  $R^2 = 0.914$ ,  $R^2 = 0.855$ ,  $R^2 = 0.877$ , and  $R^2 = 0.774$  for CK,  $\text{GA}_3$  treatment at  $100 \text{ mg L}^{-1}$ , IAA treatment at  $300 \text{ mg L}^{-1}$ , and combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$ , respectively (Figure 4).



**Figure 4.** Dynamics and rate of wood cell development with different phytohormones treatments. (A) Development of the total wood layers. (B) Rate of cell production.

The pattern in the rate of tree-ring growth was significantly changed by combination treatment AT  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  compared to CK (Figure 4). The time when the maximum rate (TMR) appeared was earliest in combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$ , at 21.40 DAT, followed by IAA treatment at  $300 \text{ mg L}^{-1}$ , at 25.40 DAT. The TMR in  $\text{GA}_3$  treatment with  $100 \text{ mg L}^{-1}$  was close to that of CK (53.90 DAT and 54.40 DAT, respectively). The maximum rate (MR) in combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  was about twice as much as in the CK (0.21 and 0.11, respectively), followed by IAA treatment at  $300 \text{ mg L}^{-1}$  (0.16) and  $\text{GA}_3$  treatment at  $100 \text{ mg L}^{-1}$  (0.07). Similar to the trend of MR, the average rate (AR) was highest in combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$ , followed by IAA treatment at  $300 \text{ mg L}^{-1}$ , CK, and  $\text{GA}_3$  treatment at  $100 \text{ mg L}^{-1}$  (0.13, 0.10, 0.07, and 0.04, respectively) (Table 3).

**Table 3.** Parameter estimates of the model for the total wood layers produced during treatments.

| Treatment                                                           | TMR (DAT) | MR   | AR   |
|---------------------------------------------------------------------|-----------|------|------|
| CK                                                                  | 54.40     | 0.11 | 0.07 |
| $\text{GA}_3$ $100 \text{ mg L}^{-1}$                               | 53.90     | 0.07 | 0.04 |
| IAA $300 \text{ mg L}^{-1}$                                         | 25.40     | 0.16 | 0.10 |
| IAA + $\text{GA}_3$ $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$ | 21.40     | 0.21 | 0.13 |

TMR: time (days after treatment) when maximum rate appeared. MR: maximum rate. AR: average rate.

#### 4. Discussion

##### 4.1. IAA and $\text{GA}_3$ (1:1) Combination Treatment Best Induced Tree-Ring Growth

Increased  $\text{CO}_2$  would promote the accumulation of phytohormones in plants. In previous studies, endogenous phytohormones such as IAA and  $\text{GA}_3$  reached levels of about  $300 \mu\text{g g}^{-1}$  FW [6,7], or much lower at about  $10 \text{ ng g}^{-1}$  [33]. Choosing the concentrations of exogenous phytohormone to apply in treatment based on endogenous phytohormone concentrations is not appropriate. Concentrations in studies on exogenous phytohormones application in plants have typically been much higher than endogenous phytohormones, at about  $200 \text{ mg L}^{-1}$  [33,34]. Previous studies have shown that the concentration of exogenous IAA and GAs that promotes wood formation is  $100 \text{ mg L}^{-1}$  for foliar application [12,30]. The 1:1 proportion of combination treatment with IAA and GAs may better promote tree-ring growth [30,31,35] than IAA and  $\text{GA}_3$  treatment alone. However, previous research has also shown that high IAA/low GA combination treatment favors xylem formation in *Populus robusta* [30]. To obtain the best proportion for combination treatment in terms of wood formation, particularly inducing latewood formation, in this study the combination treatment was designed as IAA+ $\text{GA}_3$  at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  (1:1),  $300 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  (3:1), and  $500 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  (5:1), and corresponding single phytohormone treatments were also considered. The appropriate proportion of IAA+  $\text{GA}_3$  combination treatment was 1:1 ( $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$ ). It is well known that the secondary xylem (wood) is derived from the vascular cambium [36]. Combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  accelerated the rate of wood cell production and caused the MR to appear earlier through increasing cambium division so that tree-ring growth was promoted. Combination treatment at  $100 \text{ mg L}^{-1} + 100 \text{ mg L}^{-1}$  significantly promoted LW formation but not EW in this treatment because the experiment started too late to affect EW formation. In previous studies, it has been reported that auxin and GA induce the division of the cambium [37]. Results from *Eucalyptus grandis* and *Populus tremula* L.  $\times$  *tremuloides* Michx showed that the application of GA would stimulate polar auxin transport [28,38]. These reports indicate that  $\text{GA}_3$  may strengthen the function of IAA in inducing cambium activity in the present study. Similar results were also found in *Populus robusta* [30] and conifers [31,35] after the application of GA and IAA together, but the dose of each phytohormone was different between different species. The tree-ring growth was inhibited by  $\text{GA}_3$  treatments in the present study, especially at  $100 \text{ mg L}^{-1}$ , because the cambium activity was inhibited during the experiment. These results are similar to the findings in *Populus simonii*  $\times$  *P. nigra* [12] and carrots [39]. However, the mechanism remains unclear.

#### 4.2. Effect of Phytohormone Application on Wood Cell Morphology

IAA treatment at  $500 \text{ mg L}^{-1}$  significantly increased the WRT of LW in the present study, similar to findings in maize (*Zea mays* L.) [40]. Auxin treatment was also able to induce the deposition of the latewood cell wall in conifers in this study. Similarly, studies have shown that IAA is one of the most important plant hormones in the process of secondary cell wall deposition [16]. In this study, the enhanced deposition of the cell wall in LW resulted in a significantly higher TWW in the IAA treatment at  $500 \text{ mg L}^{-1}$ . These results confirm the function of IAA in wood formation [14,17] and indicate that IAA participates in inducing the differentiation of latewood. The WRT of EW was significantly increased by  $\text{GA}_3$  treatment at  $300 \text{ mg L}^{-1}$ , which was similar to reports that cell wall thickness and deposition are enhanced by GAs [24,41]. This could have been caused by the induced expression of secondary wall biosynthesis-related genes [42,43]. The wood cell morphology in the combination treatment was similar to the corresponding IAA treatment alone or  $\text{GA}_3$  treatment alone. Only one of the two phytohormones in the combination treatment was related to wood cell morphology, instead of requiring crosstalk between them.

#### 4.3. High IAA/low $\text{GA}_3$ Combination Treatment and High $\text{GA}_3$ Treatment Led to the Death of Treated Seedlings

Finally, only the combination treatment at 1:1 promoted tree-ring growth and kept the treated seedlings alive. High IAA/low GA combination treatment at 3:1 and 5:1 in the present study and  $\text{GA}_3$  treatment at  $500 \text{ mg L}^{-1}$  resulted in the death of treated seedlings. In the present study, via the high  $\text{GA}_3$  treatment and combination treatment with high IAA/low  $\text{GA}_3$ , we aimed to find the threshold of concentration and proportion and then to find the best proportion for the combination treatment. These results indicate that the treatments mentioned above cannot be used in the future. Previous research has shown that IAA treatment significantly increases abscisic acid (ABA) content [44]. The application of GAs could promote the accumulation of IAA [38]. Hence, it can be assumed that combination treatments with high IAA/low  $\text{GA}_3$  cause a higher endogenous ABA content, increasing to a lethal dose, through inducing IAA accumulation with GAs help than corresponding IAA treatment alone. Even though there was a negative effect between GAs and ABA [45,46], the GA content may not have been enough to inhibit the lethal effect in high IAA/low  $\text{GA}_3$  treatment (3:1 and 5:1) in the present study. For the 1:1 combination treatment, the increased ABA content was not high enough to cause the death of seedlings because of the relatively low IAA and inhibition of  $\text{GA}_3$  toward ABA. The death of the treated seedlings in the  $\text{GA}_3$  treatment at  $500 \text{ mg L}^{-1}$  could have been caused by the crosstalk between  $\text{GA}_3$  and IAA, leading to lethal ABA accumulation, or simply have been caused by the high  $\text{GA}_3$  itself. All the hypotheses mentioned above require further study in the future.

### 5. Conclusions and Implications

This study is the first to demonstrate the synergistic effect of IAA and  $\text{GA}_3$  on tree-ring growth of hybrid larch, especially latewood formation, and the optimal hormone ratio conducive to its growth is obtained.

Exogenous IAA and  $\text{GA}_3$  were applied together to investigate the effects of combination treatment with these two phytohormones on tree-ring growth of hybrid larch in the study. We found that the IAA +  $\text{GA}_3$  combination treatment at  $100 \text{ mg L}^{-1}$  +  $100 \text{ mg L}^{-1}$  (1:1) was the most appropriate to promote tree-ring growth in hybrid larch seedlings. Specifically, it increased the rate of wood cell production, especially the LW, by inducing cambium activity to promote tree-ring growth. The effect of a single phytohormone treatment alone with IAA on wood formation was not steady and even inhibited it at  $100 \text{ mg L}^{-1}$   $\text{GA}_3$  treatment. These results indicate that the wood formation of hybrid larch may be affected by a complex phytohormone network. In conclusion, the application of IAA and  $\text{GA}_3$  together promoted tree-ring growth, and the content and proportion of endogenous

phytohormones should be regarded as important indicators in improving the management of hybrid larch clonal plantations.

Under future climate change, the impact of elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>) on plants is to disturb tree growth through plant hormone channels [1–3,6]. Therefore, an in-depth understanding of the synergistic mechanism of hormones in tree growth can help to understand the response of trees to future climate change and is of great significance for sustainable forest development. In addition, this progress also provides a reference for high-quality seedling cultivation of trees. Specifically, during the seedling stage of hybrid larch, the combined application of IAA and GA<sub>3</sub> (1:1) can promote the growth of hybrid seedlings and shorten the seedling period to meet the afforestation requirements as soon as possible. These results are of great significance in theory and practice for improving the management model of larch plantations.

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