




## Article

# Comparing Rooting Ability and Physiological Changes of Two *Eucommia ulmoides* Improved Varieties

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**Abstract:** *Eucommia ulmoides* (*E. ulmoides*) is a significant national strategic resource in China. It is a natural high quality rubber resource, with great development potential. We found large differences in rooting ability during adventitious root (AR) formation in two *E. ulmoides* improved varieties. Therefore, we used two improved varieties of *E. ulmoides*, ‘Huazhong 6’ (H6, with rooting rate 85.3%) and ‘Huazhong 8’ (H8, with rooting rate 22.5%) to explore the cutting rooting mechanism. In this study, we mainly determined the morphological development process of *E. ulmoides* cutting rooting, and compared the rooting-related indexes of the two improved varieties, and the changes in physiological indexes closely related to rooting, which include endogenous hormones, oxidases and nutrients in the phloem of the basal stem. The results showed that indole-3-acetic acid (IAA), zeatin riboside (ZR), IAA/ZR and indoleacetic acid oxidase (IAAO) were the key factors that caused big differences in rooting ability between the two *E. ulmoides* improved varieties. The increase in endogenous hormone IAA content and IAA/ZR value were necessary for the formation of AR. The increase in IAA content was beneficial to AR formation. The activity of IAAO was significantly negatively correlated with the rooting ability of the *E. ulmoides* cuttings. The high IAAO activity of the H8 cuttings led to the consumption of IAA. Although the content of IAA increased, the rooting conditions were not reached. The accumulation of nutrients before rooting also has an important effect on rooting; it is easy for cuttings to root when the carbon-nitrogen ratio (C/N) value is high. This research provides an improved understanding of the cellular and physiological underpinnings of the AR process in woody plants. In addition, it provides a theoretical basis and foundation for subsequent research on *E. ulmoides* cuttage technology.

**Keywords:** *Eucommia ulmoides* Oliv; AR formation; endogenous hormones; oxidases; nutrients



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## 1. Introduction

*Eucommia ulmoides* Oliv (*E. ulmoides*), a single family, single genus, single species and dioecious, is the unique national strategic reserve resource in China [1]. *E. ulmoides* is one of the medicinal plants that have had a long history of success in traditional medicine and has special value. Extract preparations from *E. ulmoides* have had vasodilatory effects, and have been used in antihypertensive pharmacological research [2]. However, difficulties have been encountered in the traditional seed reproduction of *E. ulmoides*. Being a dioecious species, excellent traits are unstable, leading to prolonged seed dormancy, irregular fruiting, long juvenile periods, etc. Vitro plant regeneration technology could ensure the stable excellent shape of the parents and a high reproduction coefficient, but had drawbacks in

actual production, due to complex operation, high technical requirements, etc. [3]. Similarly, the use of grafting reproduction technology also required a lot of labor costs. Cutting propagation had the characteristics of stable genetic characters, a short seedling raising period, simple operation and low cost, so it played a key role in genetic improvement and commercial production of *E. ulmoides* [4]. The formation of adventitious root (AR) is necessary for the cutting propagation of *E. ulmoides*, but there are genetic specificities between different cultivators of the same tree species, and the rooting abilities are also quite different [5]. The special rooting agent for *E. ulmoides* invented by our research group can significantly increase the rooting rate of most *E. ulmoides* varieties. The rooting rate of H6 cuttings treated with the special rooting agent is stabilized at more than 85%, and the rooting rate of other varieties can reach more than 75%. However, the rooting rate of H8 is only 20–30%; finding the reason is of great significance to the breeding and application of *E. ulmoides*.

Studies have shown that the content and ratio of plant endogenous hormones have significant roles regulating the formation of ARs [6,7], while auxin has a positive effect on the induction of ARs [8,9]. Peroxidase (POD), polyphenol oxidase (PPO) and indole acetic acid oxidase (IAAO) are considered to be the three oxidases most closely related to the formation of ARs, and their activity changes have obviously promoted or inhibited effects on cutting rooting [4,10,11]. When rooting, cuttings need to consume a lot of nutrients and energy, and sufficient nutrients are a necessary condition for AR formation [11,12]. In general, it is easier for cuttings to take root when the C/N value is higher, otherwise it is difficult [4]. Current studies pay less attention to the rooting mechanism of softwood cuttings such as *E. ulmoides*, and there are fewer research reports on related physiological indexes in the cutting process of *E. ulmoides*. According to the compared differences in physiological and biochemical indexes during the rooting process of *E. ulmoides* cuttings, this result can provide a theoretical basis for the further innovation of cutting propagation technology and the research on the cutting rooting mechanism of *E. ulmoides*.

## 2. Materials and Methods

### 2.1. Test Materials

The cuttings were taken from 3-year-old grafted improved varieties of *E. ulmoides*, 'Huazhong 6' (H6) and 'Huazhong 8' (H8), cultivated from the Eucommia Engineering Research Center of State Forestry and Grassland experimental base (34°50' N, 112°33' E) located in the Mengzhou, Henan province, China. The semiligninized spring shoots with a length of 10–15 cm were selected as cuttings, and top tender shoots 2–3 mature leaves were retained. The cuttings should be picked manually from the base with no scissors.

### 2.2. Experimental Design

The cuttings (currently used and prepared) from the two *E. ulmoides* improved varieties were dipped in the special rooting agent (patent application number: CN202011236585.9) for 10 s, and each variety was inserted into the cutting bed that was disinfected with 40% carbendazim wettable powder 500 times before cutting. The cutting bed was 8 m in length and 1.5 m in width, and coarse river sand to an 0.15 m thickness was spread in the lower part, and covered with an 0.15 m matrix (turfy soil:perlite = 3:1) in the upper. Every two days, 20 cuttings were randomly selected to observe the phenotypic changes, and 3–4 cuttings with the same growth state were selected at the same time. The stem segments with the length of 1–3 cm at the base of cuttings were put into a 70% FAA (Formaldehyde–acetic acid–ethanol fixative) fixation solution and preserved at low temperature without light in the laboratory. The anatomical observation of rooting was carried out, and the rooting was observed by section until rooting. The cuttings which were not treated with the rooting agent were collected as the first sample (0 day). The other samples were collected at 6, 12, 18, 22, 26, 32, 39, 46, 53 days after their insert into the cutting bed and their microscopic morphological changes were observed under stereomicroscope. In order to determine endogenous hormones, oxidase and nutrients, samples were collected 3 times

each time and put into 15 mL centrifuge tubes, each test tube sample with a weight of 4–5 g. All treated tissue samples were immediately frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  for subsequent analysis.

### 2.3. Growth Index Statistics

The morphological changes of the cutting incision and the base of the cutting were observed regularly after insertion. The rooting rate, average number of roots and average root length of cuttings (accurate to 0.01 cm) were calculated after cutting for 60 days.

### 2.4. Determination of Physiological Index

High performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) was used to determine the content of endogenous hormones, including: IAA, gibberellic acid ( $\text{GA}_3$ ), abscisic acid (ABA), ZR. The determination parameters were as follows: BEH C18 column ( $2.1 \times 100\text{ mm}$ ,  $1.8\text{ }\mu\text{m}$ ), mobile phase A is 0.1% formic acid aqueous solution, B is acetonitrile, linear gradient elution, flow rate  $0.25\text{ mL/min}$ , injection volume  $5\text{ }\mu\text{L}$ , column temperature  $40\text{ }^{\circ}\text{C}$ . The mass spectrometry conditions are: electrospray ionization source (ESI) is at positive and negative ion ionization mode, the ion source temperature is  $500\text{ }^{\circ}\text{C}$ , the ion source voltage is  $5\text{ }500\text{ V}$ – $4\text{ }500\text{ V}$ , the curtain gas is 30 psi and the atomization gas and auxiliary gas are both 50 psi. Multireaction monitoring mode (MRM) was used for scanning. For oxidase activity (IAAO, PPO, POD), soluble sugar, soluble protein content determination tests and calculation methods, refer to Li et al. [13]. The total nitrogen content was determined by the Kjeldahl method. Each determination was treated with 3 biological replicates.

### 2.5. Data Analysis

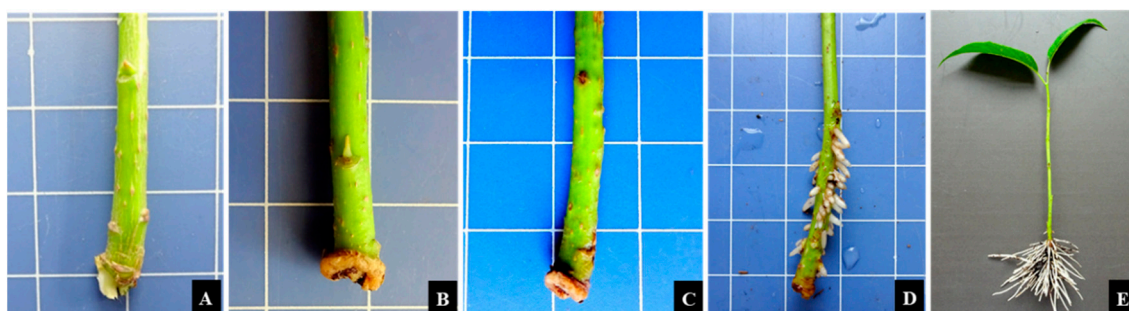
Microsoft Excel 2016 was used for data collation, SPSS20.0 for Windows SPSS, Chicago, IL, USA was used for the analysis of variance (ANOVA,  $p \leq 0.05$ ), and Origin 2020 software was used to make charts.

## 3. Results

### 3.1. The Rooting of Softwood Cuttings of *E. ulmoides*

#### 3.1.1. Morphological changes

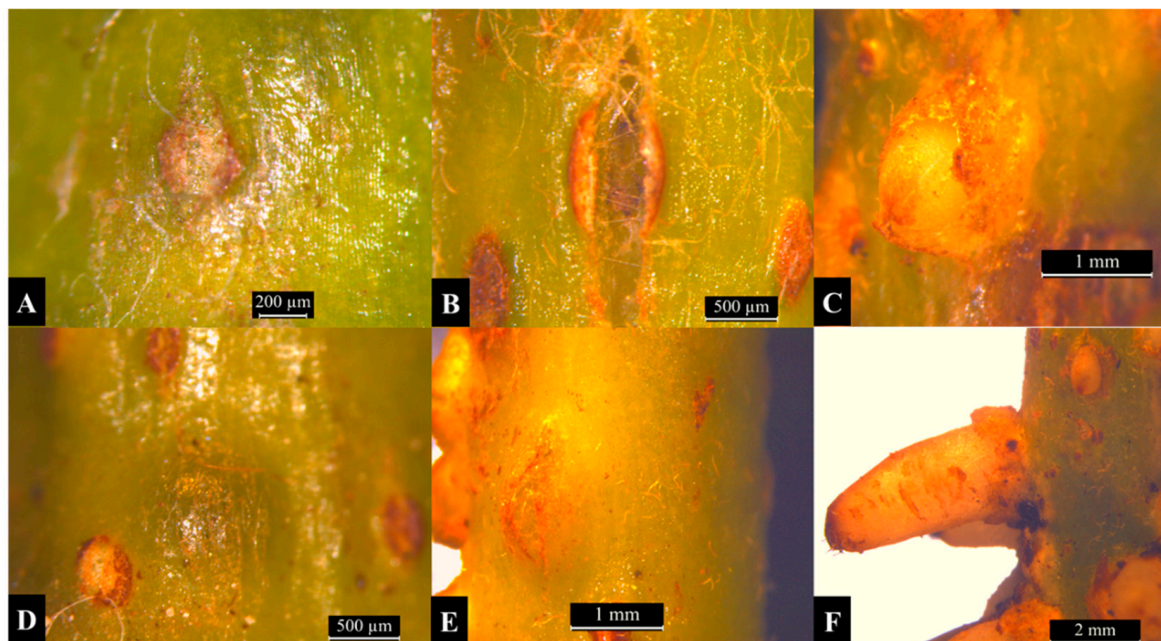
In observing the rooting of H6 softwood cuttings, it was found that in *E. ulmoides*, young AR burst from the cortex within 3 cm above the basal cut. The base of cuttings healed and merged to form a few callus (Figure 1B) after being treated with a special rooting agent for *E. ulmoides* at about 12 days. The basal cortex of the cuttings showed transparent protrusion (Figure 1C), accompanied with pore cracking, at around 22 days. Cuttings at about a month were forming a visible white AR protruding from the cortex (Figure 1D). A fortnight later, the AR entered the elongation stage (Figure 1E) and gradually formed a complete root.



**Figure 1.** Morphological changes of *E. ulmoides* cuttings in the rooting process. (A–E) The rooting situation of cutting on the 0th, 12th, 22nd, 32nd and 46th day of *E. ulmoides*.

### 3.1.2. Microscopic Morphological Changes

The rooting process of *E. ulmoides* cuttings was observed through a stereomicroscope, and it was found that the root primordium is not consistent, which is shown in Figure 2A–F. It is shown that lenticels at the base of the cuttings gradually cracked, and white AR emerged in Figure 2A–C. However, some AR emerged directly in the cortex. Firstly, dark protuberances were formed in the cortex (Figure 2D), then translucent root tips broke through the cortex (Figure 2E) and the ARs turned white during elongation (Figure 2F). The existence of lenticels relatively reduced the mechanical resistance of ARs protruding from the cortex. The process from lenticel cracking to the AR breaking through the cortex of the cuttings is mostly concentrated to 22–32 days.

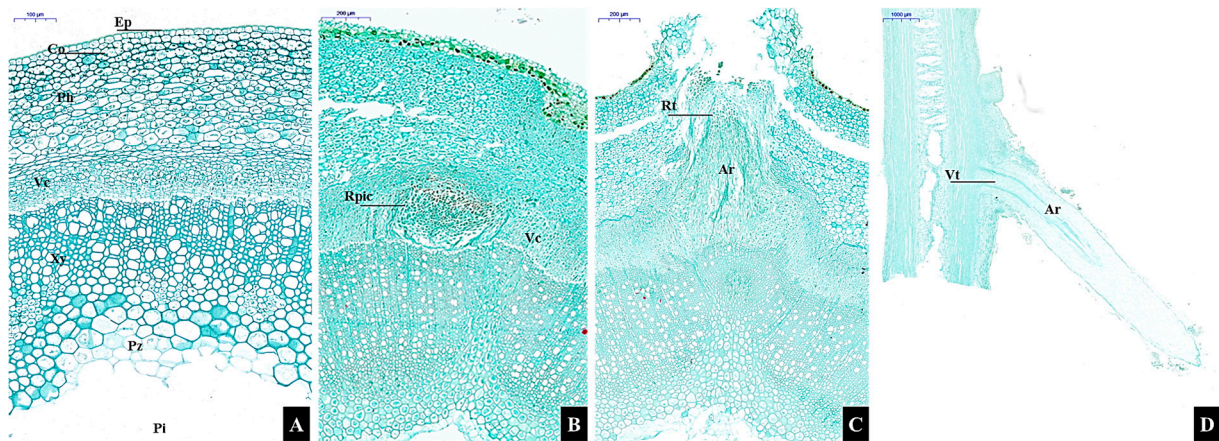


**Figure 2.** Microscopic morphological changes in the formation of the AR of *E. ulmoides* cuttings. (A–C) adventitious roots were extended from lenticels. (D–F) adventitious roots were extended from cortex.

### 3.1.3. Anatomical structure changes

It can be seen from Figure 3 that no latent root primordium was found in the cuttings of *E. ulmoides* (Figure 3A), so the root primordium of *E. ulmoides* belongs to the inducible root primordium, which mainly originates from the cambium. Through paraffin section technology, we observed that there were clumps of meristems formed by parenchyma cells with dense cytoplasm and large nuclei in the cambium at 18 days, which is a root primordium initial cell (Figure 3B). Subsequently, the root primordium further developed to form AR, which broke through the cortex (Figure 3C). At about one month, most of the AR has entered the elongation stage (Figure 3D).

Combined with the results of phenotypic changes in the rooting process of cuttings and the anatomical results of paraffin sections, the rooting process of *E. ulmoides* cuttings were divided into four stages: AR induction period (0~12 days), initiation period (12~22 days), expression period (22~32 days) and elongation period (32~53 days).



**Figure 3.** Anatomical changes in the rooting process of *E. ulmoides* twig cuttings. (A–D) The rooting situation of cutting seedlings on the 0th, 18th, 26th and 32nd day of *E. ulmoides*. Vc—Vascular cambium; Ph—Phloem; Xy—Xylem; Ve—Vessel; Ep—Epidermis; Co—Cortex; Pz—Perimedullary zone; Pi—Pith; Rpic—Root primordium initial cell; Ar—AR; Vt—Vascular tissue; Rt—Root tip.

### 3.2. Morphological Comparison of *E. ulmoides* Cuttings

After 22 days, the basal cortex of H6 cuttings protruded and split, the top of the protuberance was translucent, and the root primordium was induced inside (Figure 4A). However, there were a large number of callus in the incision at the base of H8 cuttings, and the change of the cortex at the base was not obvious (Figure 4D), indicating that callus formation significantly affects the rooting process. After 39 days of cutting, most of the H6 cuttings entered the stage of AR elongation (Figure 4B), some cuttings forming secondary lateral roots, while H8 had a small amount of rooting, most of the callus at the base of cuttings were yellowish brown (Figure 4E), and the base of some cuttings were black and even necrotic. After 53 days, most of the top tips of the cuttings were stretched out and the ARs had accelerated elongation, and the cuttings had basically grown into complete plants (Figure 4C). Although few of the cuttings of H8 formed a complete root, most of the cuttings entered a state of ‘pseudo-death’ after the callus formed, which lead to the serious phenomenon of ‘stunted seedlings’ (Figure 4F).



**Figure 4.** Morphological characteristics in rooting process of *E. ulmoides* cuttings. (A–C) The rooting situation of cuttings on the 22nd, 39th and 53rd day of *E. ulmoides* ‘Huazhong 6’. (D–F) The rooting situation of cuttings on the 22nd, 39th and 53rd day of *E. ulmoides* ‘Huazhong 8’.

The statistical rooting results are shown in Table 1. Compared with H8 and the control group, the rooting ability of H6 cuttings treated with the special rooting agent for *E. ulmoides*

was higher. The rooting rate and average rooting number of the H6 group were 85.30% and 25.6, respectively, which was 62.77% and 10.35 higher than H8, respectively. The results showed that the rooting ability of H8 cuttings was low and that it was an improved variety of *E. ulmoides* which is difficult to root by cuttings.

**Table 1.** Comparison of rooting ability of two *E. ulmoides* improved varieties <sup>1</sup>.

Material	Rooting Rate (%)	Average Length of Root (cm)	Length of Longest Root (cm)	No. of Root per Shoot	* Effect of Rooting
H8	22.53 ± 1.97 <sup>a</sup>	9.55 ± 4.54 <sup>a</sup>	13.75 ± 6.55 <sup>a</sup>	15.25 ± 3.79 <sup>a</sup>	145.64
H6	85.30 ± 2.52 <sup>b</sup>	8.41 ± 2.57 <sup>a</sup>	13.30 ± 2.36 <sup>a</sup>	25.60 ± 4.01 <sup>b</sup>	215.3
CK	21.3 ± 1.53 <sup>a</sup>	4.33 ± 1.17 <sup>c</sup>	8.3 ± 1.76 <sup>b</sup>	5.35 ± 1.68 <sup>c</sup>	23.17

<sup>1</sup> The different letters within the same column mean the significance at the 0.05 level. \* Effect of rooting = No. of root per shoot × average length of root (cm). H8: 'Huazhong 8' treated with the special *E. ulmoides* rooting agent. H6: 'Huazhong 6' treated with the special *E. ulmoides* rooting agent. CK: 'Huazhong 6' treated with water.

In this experiment, the rooting rate of H6 cuttings was higher than that of previous years, mainly due to the influence of the epidemic situation of COVID-19, which led to the late start of this cutting experiment, resulting in the overall length of cuttings and the high degree of lignification.

### 3.3. The Content and Ratio of Endogenous Hormones

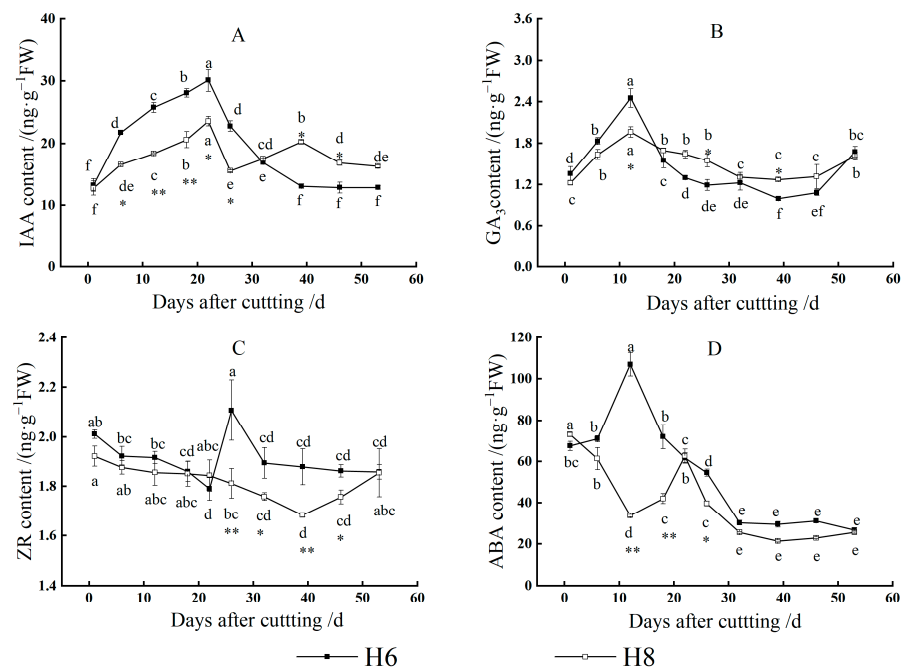
#### 3.3.1. Changes in Endogenous Hormone Content

##### Changes in IAA Content

It can be seen from Figure 5A that the IAA content of the H6 cuttings showed a trend of 'rising–falling', while the H8 had two peaks. The IAA content of the cuttings in the two groups increased significantly, and reached a peak at 22 days. Compared with the initial cuttings, the IAA content of H6 and H8 increased by 127.20% and 84.62%, respectively. At this time, a small number of cuttings had grown ARs in H6, while H8 was still forming callus. It shows that the induction of root primordium requires a high content of endogenous IAA to start, and the peak period is often accompanied by the occurrence of ARs. After 22 days, the IAA content of the H6 cuttings decreased continuously, stabilized at the initial level on the 39th day, and a second peak of H8 appeared on the 39th day. This may be because the H6 cuttings had a large number of ARs and a large amount of IAA is consumed in the elongation, which also indicates that a low concentration of IAA is beneficial to the elongation of ARs. Therefore, the low content of IAA in the H8 cuttings made it difficult to induce root primordium formation.

##### Changes in GA<sub>3</sub> Content

It can be seen from Figure 5B that the variation trend of gibberellic acid (GA<sub>3</sub>) content in the H8 and H6 cuttings was almost the same, both of which were 'rising–falling–rising'. Until 12 days, the GA<sub>3</sub> content in both varieties increased and formed a peak, although the GA<sub>3</sub> peak value of the H6 cuttings was higher than the H8 cuttings, and both the GA<sub>3</sub> and abscisic acid (ABA) contents of the H6 cuttings showed small peaks on the 12th day (Figure 5B,D), indicating that they may have synergy during this period. After 12 days, the GA<sub>3</sub> content in the H6 cuttings was slightly lower than in the H8 cuttings, and both of them rose slightly until 39 days. The variation trend of GA<sub>3</sub> content of the two improved varieties is similar, which indicates that GA<sub>3</sub> is not the main reason for the difference of rooting ability between the two improved varieties.



**Figure 5.** Changes of endogenous hormones in cutting rooting of H6 and H8 in *E. ulmoides*. **(A)** Changes in IAA content. **(B)** Changes in GA<sub>3</sub> content. **(C)** Changes in ZR content. **(D)** Changes in ABA content. IAA—Indole-3-acetic acid; GA<sub>3</sub>—Gibberellic acid 3; ZR—Zeranol riboside; ABA—Abscisic acid. The data are shown as the means  $\pm$  SEs ( $n = 3$ ). Means with the same letter are not significantly different in the same group. \* indicates significant difference between the H6 group and the H8 group at the same time. \* and \*\* indicate means at the level of 0.05 and 0.01, respectively.

#### Changes in ZR Content

As shown in Figure 5C, the ZR content of cuttings in the H6 group showed a trend of ‘decrease–rise–decrease’. The ZR content of cuttings in the H8 group changed little and decreased continuously from cutting until it increased slightly on the 39th day. Before cutting, the ZR content of H6 cuttings was higher than that of the H8 cuttings. The content of ZR in both varieties decreased continuously during the 0–22 day period. The ZR content of the H6 cuttings significantly increased to the peak at 22–26 days when the lenticels of the H6 cuttings expanded and cracked, and there were obvious protuberances at the base of the cuttings, which was a key stage of AR development, while H8 had no obvious change. All the results showed that a significant increase of endogenous ZR content in cuttings was closely related to the new young AR.

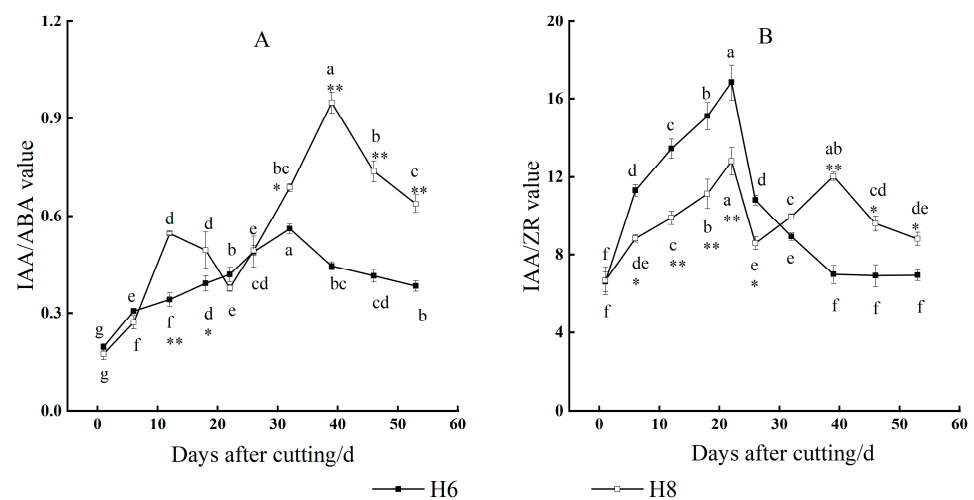
#### Changes in ABA Content

The abscisic acids (ABA) content of the H6 cuttings showed a ‘rising–decreasing’ trend, and the H8 cuttings showed a ‘declining–rising–declining’ trend (Figure 5D). The ABA content of the H6 cuttings increased until day 12 and then decreased, whereas an opposite trend can be observed in the H8 cuttings. After 22 days, both cuttings (H6 and H8) changed in the same trend. The ABA content of the H6 cuttings increased to the peak at the early rooting stage (0–12 days), which may be due to the increase in ABA content stimulated by the wound caused by cuttings detaching from the mother tree, while the increase in ABA content could reduce the damage of abiotic stress to cuttings. The ABA content of the H8 cuttings only increased from 12 to 22 days, indicating that the response of H8 to injury stimulation lagged behind. After 22 days, the ABA content of cuttings in the two groups decreased rapidly and gradually stabilized at 32 days, indicating that a low ABA content was beneficial to cuttings root elongation.

### 3.3.2. Changes of Endogenous Hormones Ratio

#### Changes in IAA/ABA

During the whole rooting process of the cuttings, there was a great difference in the change trend of IAA/ABA values among the different improved varieties (Figure 6A). The IAA/ABA value of the H6 cuttings showed a ‘rising–decreasing’ trend, while H8 showed a double-peak curve, and the two peaks appeared on the 12th and 39th day, respectively, corresponding to the valley value of ABA, and the second peak of IAA in the H8 cuttings, respectively. The IAA/ABA value of H8 fluctuated much more than H6, indicating that a more stable IAA/ABA value was more beneficial to the induction and occurrence of AR in cuttings in the rooting process.



**Figure 6.** Changes of endogenous hormone content in cutting rooting of H6 and H8 in *E. ulmoides*. (A) Changes in IAA/ABA. (B) Changes in IAA/ZR. The data are shown as the means  $\pm$  SEs ( $n = 3$ ). Means with the same letter are not significantly different in the same group. \* indicates significant difference between the H6 group and the H8 group at the same time. \* and \*\* indicates means at the level of 0.05 and 0.01, respectively.

#### Changes in IAA/ZR Value

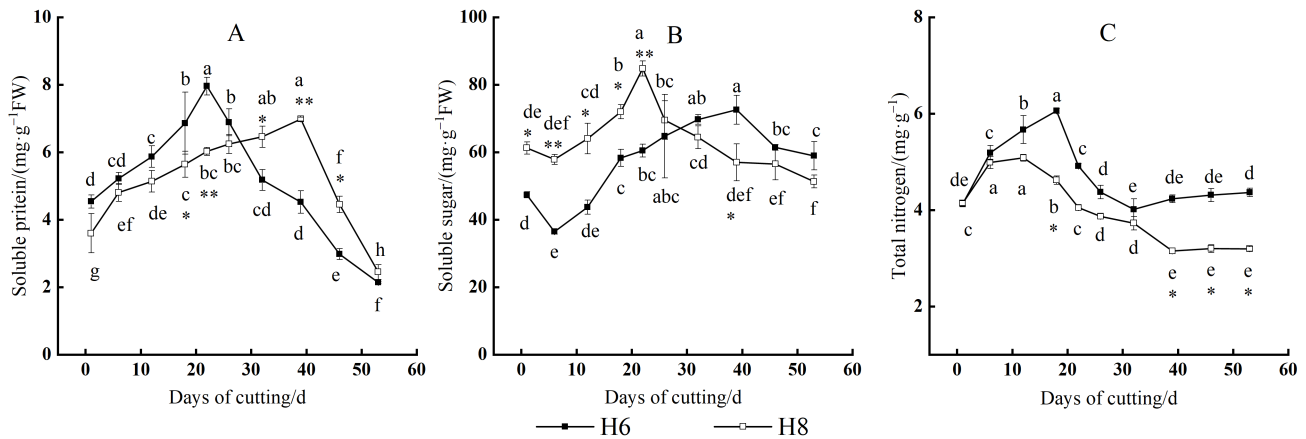
The IAA/ZR value and IAA content of the two varieties have a similar changing trend, which also indicates that IAA plays a key role in the rooting of *E. ulmoides* cuttings (Figure 6B). The peak value of IAA/ZR of the H6 cuttings was higher than that of the H8 cuttings, since the increase range of the IAA content and the decrease range of the ZR content of the H6 cuttings were higher than of the H8 cuttings during the 0~22 day period. The peak value of IAA/ZR appeared in the critical period of rooting, indicating that a higher value of IAA/ZR was beneficial to the rooting of *E. ulmoides* cuttings. After 22 days, the IAA content of H6 continued to decrease, and the ZR content increased rapidly and then decreased slowly. After 39 days, the IAA content of H8 formed a second peak, while the ZR content formed a valley. Therefore, the IAA/ZR value of H8 formed a second peak on the 39th day, and the IAA/ZR value of H6 decreased more obviously.

#### 3.4. Changes in Oxidase Activity

At the beginning of cutting, the peroxidase (POD) and indoleacetic acid oxidase (IAAO) activity of the H8 cuttings were higher than the H6, and the polyphenol oxidase (PPO) activity was lower than the H6. During the rooting process of *E. ulmoides* cuttings, the changes in POD and PPO activities of the two improved cuttings were similar, showing a ‘rising–decreasing’ trend (Figure 7A, B). There was no significant difference between them, indicating that POD and PPO were not the main reason for the difference in rooting ability. The IAAO activity of the H6 cuttings decreased significantly, while the H8 decreased and then increased. The large decrease in H6 IAAO activity corresponds to the large increase



in IAA content (Figure 7C). During the whole rooting process, the IAAO activity of H8 was higher than that of H6, which indicates that the high IAAO activity of the H8 cuttings resulted in a low IAA content in the process of AR formation, which was disadvantageous to cuttings rooting.



**Figure 7.** Dynamic changes in oxidase activity during cutting rooting of H6 and H8 in *E. ulmoides*. (A) Changes in POD activity. (B) Changes in PPO activity. (C) Changes in IAAO activity. POD—Peroxidase; PPO—Polyphenol oxidase; IAAO—Indoleacetic acid oxidase. The data are shown as the means  $\pm$  SEs ( $n = 3$ ). Means with the same letter are not significantly different in the same group. \* indicates significant difference between the H6 group and the H8 group at the same time. \* and \*\* indicate means at the level of 0.05 and 0.01, respectively.

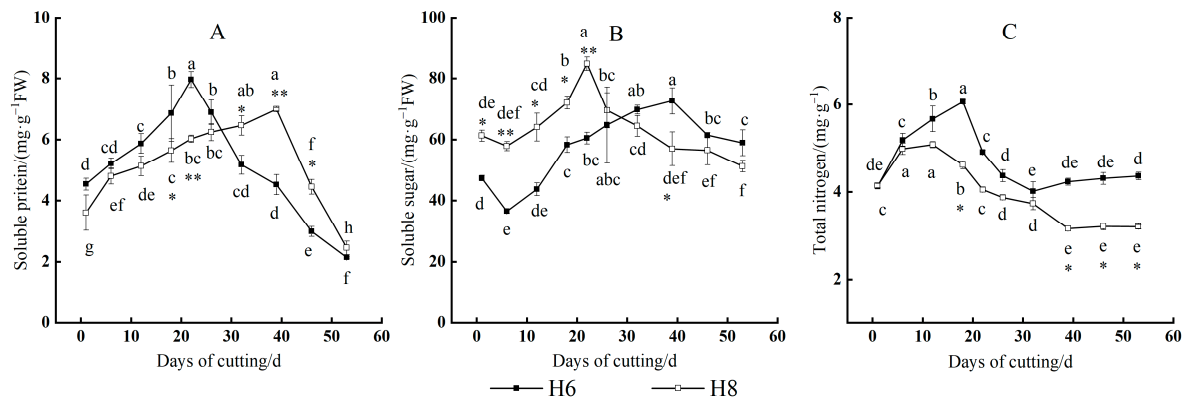
### 3.5. Changes in Nutrient Content and C/N Value

#### 3.5.1. Changes in Nutrient Content

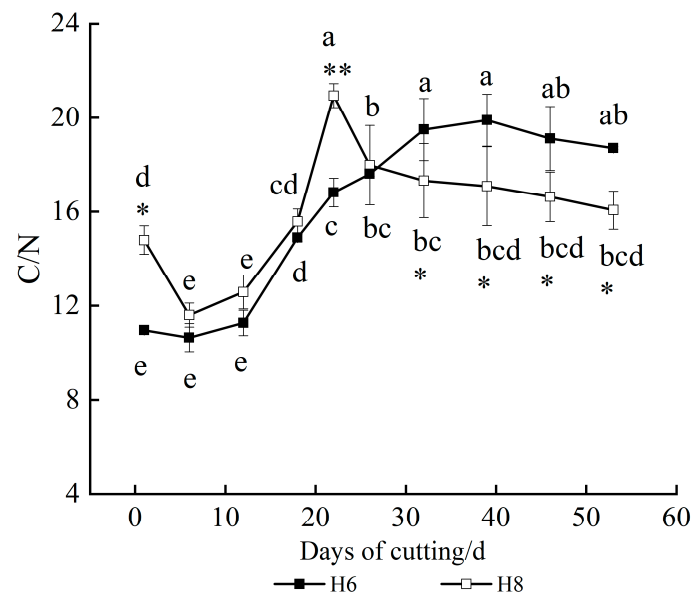
The content of soluble protein and total nitrogen in the cutting of the two improved varieties showed an ‘increasing–decreasing’ trend, and the soluble sugar content initially decreased slightly, then H8 and H6 increased to the peak on the 22nd and 39th day, respectively (Figure 8). Before cutting, the soluble protein content of the H6 cuttings was higher than the H8, while the soluble sugar content was lower than the H8 and the total nitrogen content were the same. The results showed that there were differences between the soluble protein and soluble sugar before cutting. The induction and initiation of ARs of the H6 were completed in 26 d, which was in the stage of a large number of AR development. At this stage, the content of soluble protein in H6 was higher than in H8, while the content of soluble sugar was significantly lower than in H8. The total nitrogen content of the cuttings in the two improved varieties increased and then decreased and the total nitrogen content in the H6 cuttings was always higher than H8 during the whole rooting process. Nitrogen compounds are the raw materials for the synthesis of soluble proteins. The nitrogen compounds in the H8 cuttings may synthesize soluble protein slowly due to insufficient supply, so the peak value of soluble protein appears later, which may be an important factor for the low rooting rate of the H8 cuttings.

#### 3.5.2. Change in C/N Value

The overall change in the C/N value in both *E. ulmoides* improved varieties cuttings basically showed same trend, and both increased to varying degrees during the rooting period (Figure 9). During the 6–22 day period, the C/N value level of H8 was always higher than H6, and formed a peak at day 22. This was mainly related to the higher soluble sugar content of H8. After 26 days, the C/N level of H6 was higher than H8, and the cuttings were in the elongation period of rooting. This showed that the increase in C/N value is beneficial to rooting, and the C/N value is positively correlated with the rooting ability of cuttings.



**Figure 8.** Changes in nutrient content in the cutting rooting of H6 and H8 in *E. ulmoides*. (A) Changes in soluble protein content. (B) Changes in soluble sugar content. (C) Changes in total nitrogen content. The data are shown as the means  $\pm$  SEs ( $n = 3$ ). Means with the same letter are not significantly different in the same group. \* indicates significant difference between the H6 group and the H8 group at the same time. \* and \*\* indicate means at the level of 0.05 and 0.01, respectively.



**Figure 9.** Changes in C/N value in cutting rooting of H6 and H8 in *E. ulmoides*. The data are shown as the means  $\pm$  SEs ( $n = 3$ ). Means with the same letter are not significantly different in the same group. \* indicate significant difference between the H6 group and the H8 group at the same time. \* and \*\* indicate means at the level of 0.05 and 0.01, respectively.

#### 4. Discussion

First of all, we recorded the phenotypic changes by periodic sampling and observed the slice anatomy, thus determining the four key periods of the rooting process of *E. ulmoides* cuttings, and then determined the sampling period of endogenous hormones, oxidases, nutrients and other indicators for following studies on the low rooting rate of cutting of two improved varieties of *E. ulmoides*.

##### 4.1. The Endogenous Hormones of Cutting Rooting

Indole-3-acetic acid is a key hormone in the process of plant growth and development. It can not only regulate cell division and promote cell elongation, but also affects the morphology of individual plants and their organs with the concentration gradient of their distribution [14–16]. In a study of many tree species, it was found that the content of IAA increased during the critical period of AR formation, which significantly promoted the occurrence of ARs [6,17]. Naoki et al. [18] found that the content of IAA in high rooting

rate eucalyptus (*Eucalyptus globulus*) was twice as much as low rooting rate eucalyptus varieties. The IAA level of apple (*Malus domestica*) cuttings increased continuously during the induction period (0–72 h) and start-up period (72–120 h), and reached the peak at 120 h. After the elongation period (120–168 h), it decreased steadily [19], which was consistent with the results of this study. The IAA content of the cuttings of the two improved varieties of *E. ulmoides* increased continuously during the induction and start-up periods (0–22 days), and reached the peak at 22 days. The IAA level of H6, which is an easy-to-root variety, was significantly higher than that of H8, which is a difficult-to-root variety. It is suggested that IAA is the most important endogenous hormone regulating the AR formation, and the content of IAA can be used as one of the important indexes to judge the ability of cutting rooting of improved varieties of *E. ulmoides* [20].

In recent years, there have been different conclusions about the effect of GA<sub>3</sub> on the rooting of cuttings. Some studies suggested that GA<sub>3</sub> can reduce the rooting rate by reducing the number and length of ARs in two ways, which are inhibiting the formation of root primordia and hindering the formation of AR [21,22]. However, in a study of *Rhododendron* (*Rhododendron scabrifolium*), it was found that an increase in the GA<sub>3</sub> content of cuttings was positively correlated with the induction of callus and the formation of AR [23]. In this study, it was found that the GA<sub>3</sub> content of cuttings in both varieties increased at the initial stage of cutting (0–12 days), and continued to decrease after 12 days. The results showed that the increase of GA<sub>3</sub> content was conducive to the formation of root primordium, and the decrease of GA<sub>3</sub> content was beneficial to the growth and development of AR, which was consistent with the results of tree species such as neem (*Chukrasia tabularis*) and Nanjing Tilia (*Tilia miqueliana*) [6,17]. During the rooting period, the GA<sub>3</sub> content of the two varieties of cuttings changed in the same trend and GA<sub>3</sub> may be not the main reason for the difference in rooting ability between improved varieties.

There are also controversies in the current research on the relationship between ZR and cutting rooting [24]. Some studies have suggested that ZR inhibits the rooting of cuttings, low content of ZR is conducive to root primordium differentiation and a high content of ZR promotes AR formation [22]. It is also believed that ZR has no obvious effect on root primordia, but plays an important role in the further growth and development of AR [25]. The results of this study were consistent with the former conclusion. The effect of ZR was different in each rooting stage of the *E. ulmoides* cuttings. The ZR content decreased continuously in the early stage of rooting (0–22 days), which may be related to the consumption of endogenous ZR in the process of root primordium differentiation and callus formation [26]. In plants, the main synthesis site of ZR is the root tip meristem, which transports it to the above-ground organs through ducts in order to exert its physiological regulatory role while higher concentrations of endogenous ZR are required during the growth and development primordia [27]. Therefore, after the formation of ARs in the H6 cuttings, a large number of young roots synthesized ZR, resulting in a significant increase in endogenous ZR content (22–26 days), and the rapid elongation of ARs in the later stage also consumed a certain amount of ZR, so the ZR content decreased in the later stage. The significant increase of ZR content can also be regarded as a sign that the cuttings of *E. ulmoides* began to take root in large numbers. The change in ZR content was not obvious because there was no obvious rooting phenomenon in H8. This also shows that a low content of ZR is beneficial to the induction of root primordium, but the expression stage of ARs requires a high content of ZR to participate. This is similar to the results of *Sapindus mukorossi*, and *Acer truncatum* [22,28].

Abscisic acid is the major stress hormone that coordinates plant growth, development and abiotic stress responses which can enhance the stress resistance of plants [29]. Abscisic acid has a significant inhibitory effect on the formation of ARs of cuttings [30]. In this study, the ABA content of H6 cuttings increased significantly in the early cutting stage, which was consistent with the results of *Carya illinoensis* [31]. The ABA content of H8 cuttings decreased slightly and then increased. The difference in the ABA content of the two varieties of cuttings in the early period may be related to the genetic specificity of

the improved varieties. The GA<sub>3</sub> and ABA content of H6 cuttings both peaked on the 12th day, indicating that the two may have a synergistic effect in dealing with abiotic stress. Therefore, the increase of GA<sub>3</sub> and ABA content may be closely related to plant resistance [6].

Plant cutting rooting results from the interaction of multiple plant hormones, and by studying its ratio one can more comprehensively evaluate the effect endogenous hormones have on AR development of the cutting [32,33]. The value of IAA/ABA is closely related to the rooting ability of cuttings [21,34]. Guo Sujuan et al. [12] found that the regulation of IAA/ABA on cutting rooting is much higher than any single hormone in *Pinus bungeana* cuttings. In this study, a steady increase in the IAA/ABA value was conducive to the induction and development of the root primordium, while the IAA/ABA value of the H8 cuttings fluctuated greatly, which was not beneficial to rooting. Zeatin riboside inhibited the formation of root primordium, and IAA signaling played a positive role in regulating the complex biological system. Relatively high IAA and low ZR levels are considered to be the prerequisites for inducing and initiating ARs [35,36]. During the AR formation of *E. ulmoides* cuttings, the IAA/ZR in two group increased during the induction and start-up period, corresponding to the increase in IAA content and the decrease of ZR content, which is conducive to the formation of ARs, which was consistent with the conclusion of Wang Qing et al. [6]. The peak value of IAA/ZR appeared at the critical period of rooting, and the peak value of IAA/ZR was higher in H6. Comprehensive analysis showed that the IAA/ZR value is more representative of the rooting ability of different *E. ulmoides* varieties and the stable increase of the IAA/ABA value is conducive to the formation of AR.

#### 4.2. The Oxidase Activity and Rooting of *E. ulmoides* Cuttings

The rooting of plant cuttings involves complex physiological activities, among which POD, PPO and IAAO are considered to be the three oxidases most closely related to the rooting of plant cuttings, which play a role in different stages [37,38]. In this study, the three oxidase activities had significant differences during rooting, which can be used as an important referential to determine the rooting ability of *E. ulmoides*, which is similar to the conclusions of the study on the rooting ability of different genotypes of *Sorbus* (*Sorbus pohuashanensis*) [39]. At the initial stage of plant cutting, the wood stimulation causes the cuttings to distribute phenolic substances at the wound, which promotes the increase of POD and PPO enzyme activities [28], while exogenous hormone treatment activates the POD and PPO enzyme activities in the cuttings and regulates carbohydrate metabolism [37]. An increase in POD is a sign of the rooting ability of cuttings, is involved in auxin metabolism and is related to the induction and elongation of AR [40]. Polyphenol oxidase can catalyze phenols and IAA to form a rooting cofactor, 'IAA-phenolic acid complex', which is beneficial to the root primordium [41]. The changes in POD and PPO activities of the cuttings in the two groups were basically the same and there was no significant difference, so it was considered that they were not the main cause of cutting rooting among improved varieties, and there were similar conclusions in the study of cutting rooting of *Populustremula* × *P.tremuloides* and grape (*Vitis vinifera*) [42,43]. Indole-3-acetic acid, which has a key role in the induction of ARs in the cuttings, can be oxidized and adjusted by IAAO; that is to say IAAO activity is closely related to the rooting of the cuttings [44]. Song et al. [45] found that the IAAO activity of easy rooting trees basically showed a downward trend after cutting, while difficult rooting trees changed in the opposite trend. Therefore, the decrease of IAAO activity increased the content of IAA, which could promote root formation. The negative correlation between IAAO activity and endogenous IAA level is also the characteristic of the rooting induction period of many tree species [32]. During the rooting process of *E. ulmoides* cuttings, the IAAO activity of H6 decreased significantly more than H8, while the increase of IAA content corresponding to H6 was significantly higher than H8, which accounts for the high IAAO activity, and is one of the important factors for the low rooting rate [46].

#### 4.3. The Nutrients and Rooting of *E. ulmoides* Cuttings

During the rooting process of cuttings, nutrients need to be accumulated before rooting and will be consumed in large quantities for rooting [4]. Nitrogen compounds are the main raw material for the synthesis of soluble protein, and the total nitrogen content of H8 was lower than that of H6, which may lead to the slow accumulation of soluble protein content of H8 and the late peak. Although the content of soluble protein and total nitrogen in H8 was lower than in H6, the content of soluble sugar in H8 was significantly higher than in H6, which may be due to the insufficient content of soluble protein and total nitrogen in the process of rooting [47]. In order to ensure the supply of rooting energy, the transformation and utilization of soluble sugar were increased. In the process of plant cuttings rooting, the carbon source and nitrogen source are closely coordinated to form a balance to meet the needs of plant growth and development. The C/N value can reflect the rooting ability of the cutting; the higher the C/N value, the stronger the rooting ability [48]. The C/N value of H6 was higher than that of H8. The change in C/N value mainly depended on the soluble sugar, indicating that a higher C/N value was beneficial to the rooting of softwood cuttings of *E. ulmoides*, which is consistent with the study of *Chrysanthemum morifolium* [49].

#### 5. Conclusions

The cutting propagation of plants is mainly assessed by the formation of AR, and the complex process of AR formation is regulated by many factors. The morphological comparison of *E. ulmoides* cuttings showed the rooting rate and root number per shoot in the H6 group were significantly higher than the H8 group. The dynamic change in endogenous hormones including IAA, ZR, ABA content and IAAO activity difference caused the difference in rooting ability of H6 and H8. The increase of endogenous hormone IAA content and the decrease of ZR content were the key factors for AR formation. The value of IAA/ZR and IAA/ABA were positively correlated with the rooting ability of the cuttings. The decreasing of IAAO activity in the rooting process is beneficial to the accumulation of IAA in the early stage, which is conducive to AR formation. However, the IAAO activity of H8 was higher during the rooting process, resulting in excessive consumption of endogenous IAA. Difference in nutritional content also played a primary role on the formation of AR, and H6, with a high C/N value, had a strong rooting ability. There were no obvious differences in the changes of GA<sub>3</sub> content, PPO and POD activities during the rooting process of the two *E. ulmoides* improved varieties of cuttings.

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## Abbreviations

IAA	Indole-3-acetic acid
ABA	Abscisic acid
GA <sub>3</sub>	Gibberellic acid
ZR	Zeatin riboside
POD	Peroxidase
PPO	Polyphenol oxidase
IAAO	Indoleacetic acid oxidase
C/N	Carbon-nitrogen ratio
AR	Adventitious root

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