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Effect of Auxins and Associated Metabolic Changes on Cuttings of Hybrid Aspen

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Abstract: In the present study, an attempt was made to induce rooting from single-node cuttings of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) with different concentrations of IAA, IBA and NAA during rooting. Among the three auxins used, NAA showed more effective induction on rooting as compared to IAA and IBA at the whole level. Thereafter, NAA was used further in experiments for anatomical and biochemical investigation. The results showed that it took 12 days from the differentiation of primordium to the appearance of young adventitious roots with NAA application. It was found that endogenous IAA, ZR and GA3 levels increased, but ABA decreased in cuttings with 0.54 mM NAA treatment. In contrast to the endogenous IAA level, NAA had negative effect on IAA-oxidase (IAAO) activity. Similarly, the decreased peroxidase (POD) activity, consistent with down-regulation of expressed levels of POD1 and POD2, was observed in NAA-treated cuttings. Whereas, NAA resulted in a higher activity in polyphenol oxidase (PPO) compared to the control cuttings. Collectively, the study highlighted that 0.54 mM NAA is efficient on rooting in hybrid aspen, and its effect on metabolic changes during rooting is discussed, which provide valuable information for propagating hybrid aspen.

Keywords: Auxin, Cutting, Hybrid aspen, Rooting

1. Introduction

One of the most important challenges in nowadays is the need to replace fossil fuel-based energy sources with renewable energy, such as woody biomass, in order to sequester atmospheric CO₂ [1,2]. One way to satisfy the increasing demand for woody biomass is through the establishment of short-rotation forest plantations [3]. For this purpose, hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.), a cross between the European aspen (*P. tremula* L.) and its North American counterpart, trembling aspen (*P. tremuloides* Michx.), has proven to be one of the most promising species for intensive pulp and biomass production due to its fast growth, cold resistance and pathogen resistance [4-6].

Although hybrid aspen is a difficult to root plant, propagation through cuttings is the most commonly used asexual method. The successful establishment from stem cuttings, however, depends upon many factors, like seasonal and age variation, portion and diameter of stem, growing media, moisture level, nutrient status and temperature etc. [7]. Specially, plant growth regulatory hormones or 'auxins' play a vital role in influencing the sprouting and survival of stem cuttings. Adventitious rooting (AR) is a complex developmental process that can be stimulated by exogenously applied auxins [8], which play a critical role on increasing initiation of the root primordium and growth via cell division [9]. In addition, the interdependent physiological stages of the rooting process are also associated with changes in endogenous auxin concentrations [10]. Some endogenous factors, hormones and/or hormonal balance, tissue maturity, expression of specific regulatory genes, have also been recognized as fundamental factors in this regeneration scenario [11-16]. However, the combined effects of auxin type, auxin concentration, and the accompanying biochemical changes on

rooting of hybrid aspen cuttings have not been reported. Moreover, the knowledge of the intricate signaling network participated by these factors during AR formation in cuttings is fragmentary [17]. The aim of the present study is to establish the rooting efficiency from cuttings of hybrid aspen under controlled conditions, to gain an insight into the process of redifferentiation by anatomical observation, and to investigate the biochemical changes, as well as the related gene expression during adventitious root development.

2. Materials and Methods

2.1. Preparation of stem cuttings

Mature stem cuttings of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) were collected in the experimental greenhouse in Northeast Forestry University. The leaves and shoot apices were excised and uniform leafless semi-hard wood cuttings (2–3 cm long and 0.5–1 cm diameter) comprising 1 node were prepared. The cuttings were dipped in distilled water for 10 min, subsequently treated with root promoting auxins at the basal end. The cuttings received distilled water (control) or treatments of IBA, IAA and NAA individually at different concentration ranging from 100–500 mg L⁻¹, respectively for a maximum of 1 h duration to determine the rooting capacity. Different combinations of above rooting hormones were also tried to see any possible synergistic effects in promoting the rooting ability from the stem cuttings. Cuttings raised in nursery bed were also used as source material for studying the effect of adventitious rooting and effect of juvenility treated with the best responding concentration. The top (apical) cut ends of the treated cuttings were sealed with paraffin wax to reduce the water loss. The nursery bed were incubated in a controlled growth chamber (28 ± 2°C and 80% relative humidity). Cuttings were harvested after 3, 6, 9, 12 and 15 days for anatomical, physiological and biochemical analysis, as well as the expression analysis of genes. Rooting percentage, number of roots per cutting, root length was recorded from cuttings after 3 weeks transferred to nursery bed.

2.2. Anatomical procedure

Three cuttings of each time point of treatment were investigated, the basal 1 cm of the cuttings (including the slanted cut) were removed and fixed for two days in formalin:alcohol:glacial acetic acid (FAA) solution (1:9:1; v/v/v) using 70% (v/v) ethanol in distilled water, after which they were dehydrated using a series of 30%, 50%, 70% and 100% (2×) ethanol, each for 30 min. The ethanol was substituted by means of a series of 30%, 50%, 70% and 100% (2×) xylol, each for 30 min, followed by infiltration of wax [18]. Embedded sections were transverse sectioned at 8 µm thickness and transferred onto glass slides. The slides were dipped twice in xylene for 5 min each and in ethanol for 5 min. The slides were then air dried and dipped in 1% safranin for 15 min, rinsed three times in deionized water to remove excess stain, dipped in 0.75% malachite green solution (with 5% acetic acid and 8.5% glycerol) for 15 s, and again rinsed three times in deionized water. Sections were then dried and mounted on slides, stained with safranin and counter stained with fast green, and photographed using a digital microscope (Axioskop, Zeiss, Germany).

2.3. Assay of endogenous hormones

Frozen tissue was ground with a mortar and pestle and hormones were extracted in 80% methanol. Samples were centrifugated at 10 000 g for 15 min at 4°C. The supernatant was collected and immediately applied to a pre-equilibrated C18 Sep-Pak cartridge [Millipore (Waters), Watford, UK], which was washed with 80% methanol solution and eluted with methanol:acetic acid (4:1, v/v). The extracts were collected and dried under N₂ [19]. The mouse monoclonal antigens, antibodies against ZR, IAA, GA₃, and ABA, and IgG-horseradish peroxidase in ELISA were purchased from the

Phytohormones Research Institute (China Agricultural University, Beijing, China). ELISA was performed on a 96-well microtitration plate. Each well on the plate was coated with 100 μ L coating buffer containing antigens against the hormones. The coated plates were incubated for 4 h at 37°C for ZR, GA₃, and ABA, and overnight at 4°C for IAA, and then kept at room temperature for 30 min. After washing four times with PBS + Tween 20 buffer (pH 7.4), each well was filled with 50 μ L of either extracts or ZR, IAA, GA₃, and ABA standards, and 50 μ L antibodies against ZR, IAA, GA₃, and ABA, respectively. The plate was incubated for 3 h at 28°C for ZR, GA₃, ABA, and overnight at 4°C for IAA, and then washed as above. 100 μ L of IgG-horseradish peroxidase was added to each well and incubated for 1 h at 30°C. The plate was rinsed five times with above PBS + Tween 20 buffer, and 100 μ L color-appearing solution containing 0-phenylenediamine and 0.008% (v/v) H₂O₂ was added to each well. The reaction progress was stopped by adding of 50 μ L 2M H₂SO₄ per well. Color development in each well was detected using an ELISA Reader (Sunrise, Tecan, Switzerland) at optical density A490. The results are the means \pm SE of at least four replicates.

2.4. IAA oxidase (IAAO) assay

The reaction mixture was made by mixing the 0.2 ml enzyme extracts, 0.78 ml of 50 mM potassium-phosphate buffer (pH 6.0), 0.01 ml of 5 mM MnCl₂, 0.01 ml of 5 mM 2,4-dichlorophenol and 0.02 ml of 2.5 g/l IAA. Assays were conducted at 25 \pm 0.5 °C for 30 min. The Salkowski reagent (2 ml) was then added and the destruction of IAA was determined by measuring the absorbance at 535 after 30 min [21]. Each value was the mean of three replicates. IAAO activity is represented by the amount of IAA degraded (μ g) starting from 1 mg initial protein in 1 h.

2.5. Polyphenol Oxidase (PPO) assay

Polyphenol-oxidase enzyme assay was made by using pyrogallol as the substrate [22]. The 1 ml reaction mixture contained 250 μ l of the enzyme extract and 0.1 M phosphate buffer (pH 6.0). Each sample was aerated for 2 min in a small test tube followed by the addition of 0.2 M catechol as the substrate. PPO activity was expressed as changes in absorbance at 420 nm min⁻¹ g⁻¹ FW.

2.6. Peroxidase (POD) assay

The activity of POD was determined according to the method of Li et al. [22] based on the oxidation of guaiacol using H₂O₂. The reaction mixture was made by mixing 0.1 ml enzyme extract, 0.01 ml 20 mM Guaiacol, 0.1 ml 50 mM H₂O₂, and 0.76 ml 2.5 mM 3,3-dimethylglutaric acid (3,3-DGA)-NaOH at pH 6.0. Peroxidase activity was determined spectrophotometrically by monitoring the formation of tetraguaiacol at 470 nm after 10 min incubation at 30 \pm 0.5 °C. Each value is the mean of three replicates.

2.7. Quantification of PCR products

Total RNA was treated with DnaseI and reverse-transcribed using PrimeScript RT reagents Kit (Takara). 20 ng of retrotranscribed RNA was amplified with the SYBR Premix Ex Taq Kit (Takara). The thermal cycling conditions were 40 cycles of 95 °C for 30 s for denaturation and 58 °C for 30 s for annealing and extension. All reactions were run in triplicate by monitoring the dissociation curve to control the dimers. Hybrid aspen gene actin was used as a normalizer, and the relative expression levels of genes were presented by 2^{- Δ CT} (Δ CT is the difference of CT between the actin and the target gene).

PCR reactions employed the following primers. POD1-F (AGAATCGGTTGTCTTGGCAGG -3), POD1-R (GGGATGAAAGCATTAGCAGCA); POD2-F (TAGCGGGGCTGATACTGTTGC), POD2-R (ATTTCTCCATGCTTCCTGTC -3), respectively.

3. Results

3.1. Effects of a range of auxin types on rooting

In the present study, although all three auxins resulted in a significant induction of adventitious roots in hybrid aspen cuttings, there were significant differences ($P < 0.05$) under different auxins with different concentrations. The cuttings treated with 0.54 mM NAA showed maximum percentage of rooting and maximum number of roots per cutting (Table 1). Among the different concentrations of IAA used for pretreatment of cuttings, the maximum percentage of rooting was obtained in cuttings treated with 0.54 mM IAA. Among the different concentrations of IBA used for pretreatment, the maximum percentage of rooting was observed in cuttings treated with 0.54 mM IBA. However, the cuttings pretreated with 0.49 mM IBA showed the highest root length as compared with other treatments. It is worth noting that higher doses of all three auxins tested further inhibited sprouting. In the case of IAA treatments, the inhibitions were more pronounced with 2.85 mM IAA, where percentage of rooting decreased by 35% compared to cuttings treated by 0.54 mM IAA (Table 1). Collectively, application of NAA at 0.54 mM brought the highest per cent rooting, numbers of rooting, and acceptable root length. Therefore, 0.54 mM NAA was used further for metabolic studies during rooting process.

Table 1 Effect of different pretreatment of IAA, IBA and NAA on rooting from single nodal cuttings of *Populus tremula* × *P. tremuloides* after 3 weeks transferred to potting medium.

Different treatments	% of rooting (Mean ±SE)	No. of roots/cutting (Mean ±SE)	Av. root length(cm) (Mean ±SE)
Control(H ₂ O)	5.80±0.45i	1.40±0.05e	1.43±0.04f
IAA 0.57 mM	35.48±0.63e	2.72±0.23cd	2.54±0.14c
IAA 1.71mM	19.13±0.38g	2.87±0.11c	2.38±0.14c
IAA 2.85mM	15.11±0.78h	2.53±0.10d	2.01±0.13d
IBA 0.49mM	42.10±0.67d	3.00±0.06bc	3.54±0.08a
IBA 1.47mM	34.34±1.38e	3.16±0.10b	2.92±0.10b
IBA 2.45mM	24.16±0.78f	2.72±0.05cd	2.45±0.04c
NAA 0.54mM	79.39±0.87a	4.08±0.28a	1.74±0.11e
NAA 1.62mM	63.73±1.09b	3.90±0.13a	1.53±0.13ef
NAA 2.70mM	52.86±0.24c	3.90±0.26a	1.50±0.28ef

Mean of 30 nodal cuttings/treatment; repeated thrice. Mean having the same letter in a column were not significantly different by Duncan's multiple comparison test ($p < 0.05$)

3.2. Morphological and anatomical observation of cuttings rooting process

The following description of the stem anatomy of hybrid aspen is based on transverse sections that were made from the basal part of cuttings, and it took 12 days from cutting to rooting treated by 0.54 mM NAA. The basal parts of the cuttings had a slanted cut surface where the NAA was applied (Figure 1a). The lower incision organization started to become loose after 6 days and the cortex of the lower incision of some cuttings showed minor cracking. The basal position of cuttings began to enlarge, subsequently the outside of the periderm near the incision thickened slightly, swelled and formed many small white protrusions after 9 days, then the tips of these protrusions bursted, finally transparent and white young adventitious roots erupted successively in all cuttings up to 12 days (Figure 1b-c). After 3 weeks, the adventitious roots grew to 3-5 cm (Figure 1d).

The stem is composed of periderm (including skin debris), cortex and secondary vascular tissues from the outside to the inside (Figure 1e). The cuttings first developed root primordium (Figure 1f),

and parenchyma cells located in the junction of cambium and pith ray cells regained meristematic capacity. The parenchyma cells split and formed a parenchyma cell mass which showed larger nuclei, closely arranged, more clearly distinct from the surrounding cells. Root primordium cells near cambium cells stained darker and showed stronger divisibility, gradually formed a group of smaller and darker staining meristematic cells, constituting meristematic tissue mass (Figure 1g). The cells connected with the root primordium around the cambium layer differentiated faster, forming wedge-shaped adventitious root primordia. These extended along the phloem-ray direction through the cortex and epidermis, ultimately extending outside the stems (Figure 1h). When they reached the periderm, the differentiation of adventitious root vascular systems was completed, by which time distinct layers had emerged, forming a root cap, meristematic zone and an elongation zone (Figure 1i).

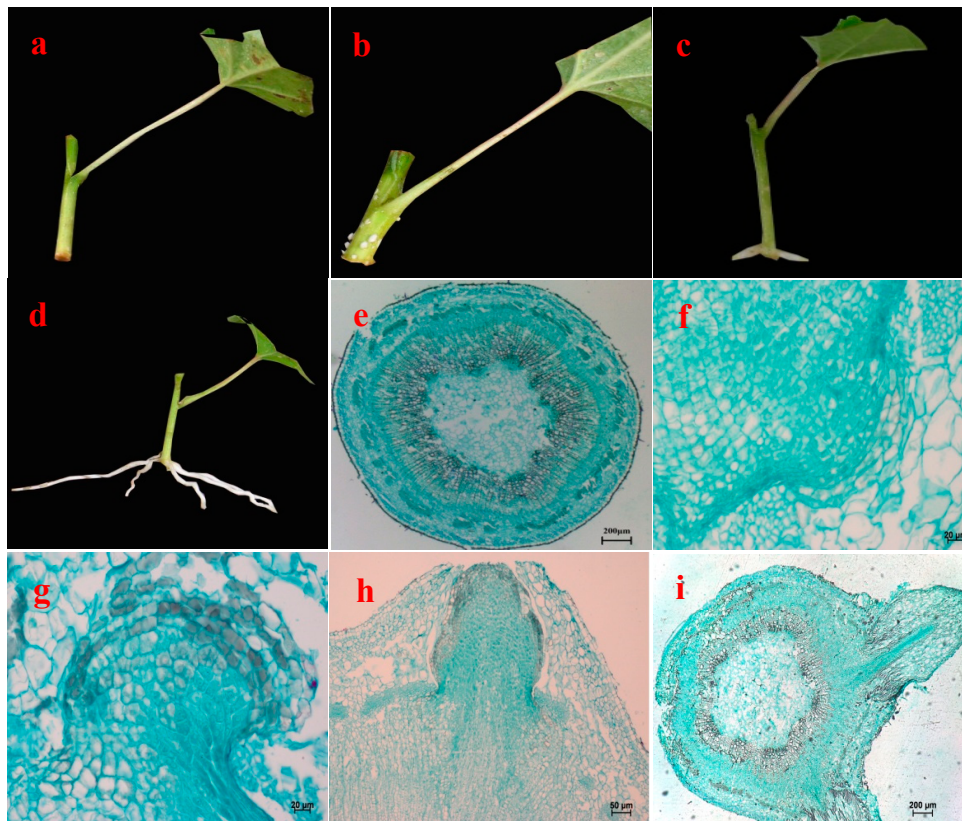


Figure 1 Morphological and anatomical observation on rooting of hybrid aspen (*Populus tremula* L.×*P. tremuloides* Michx.) cuttings. (a): Cutting after 1 days treated by NAA, (b) White dot in cutting, (c): Adventitious root emerging from stem, (d): Adventitious root, (e): Stem transection, (f): Initiative development of root primordium, (g) Continuous development of root primordium, (h), Adventitious root emerging from lenticel, Root primordium broken through cutex, (i) Adventitious root elongating growth.

3.3. Changes in the level of endogenous hormones

Higher levels of endogenous IAA were found in the rooting hypocotyls of cuttings treated with NAA in the early period of investigation (Figure 2A). At 6 d of treatment, the endogenous IAA content in the NAA-treated tissues was about 2.1 fold higher than that of controls. Thereafter, IAA level gradually decreased, up to the control level at the end of treatment. In contrast, NAA treatment resulted in a gradual decrease in ABA level during the early period examined. Around the 6th day,

the level of ABA reached a low peak (decreased by 42%). In the later period investigated, the endogenous ABA content gradually recovered to control level (Figure 2B). There was a significantly increase ($P \leq 0.05$) in endogenous ZR (zeatin riboside) level at 6 d treated (Figure 2C), and ZR content increased by 56% at 9 d in NAA-treated cuttings. As regards endogenous GA3 level, there were no changes before 6 d of NAA treatment. However, NAA significantly induced the endogenous GA3 content ($P \leq 0.05$) during 9–12 d examined (Figure 2D).

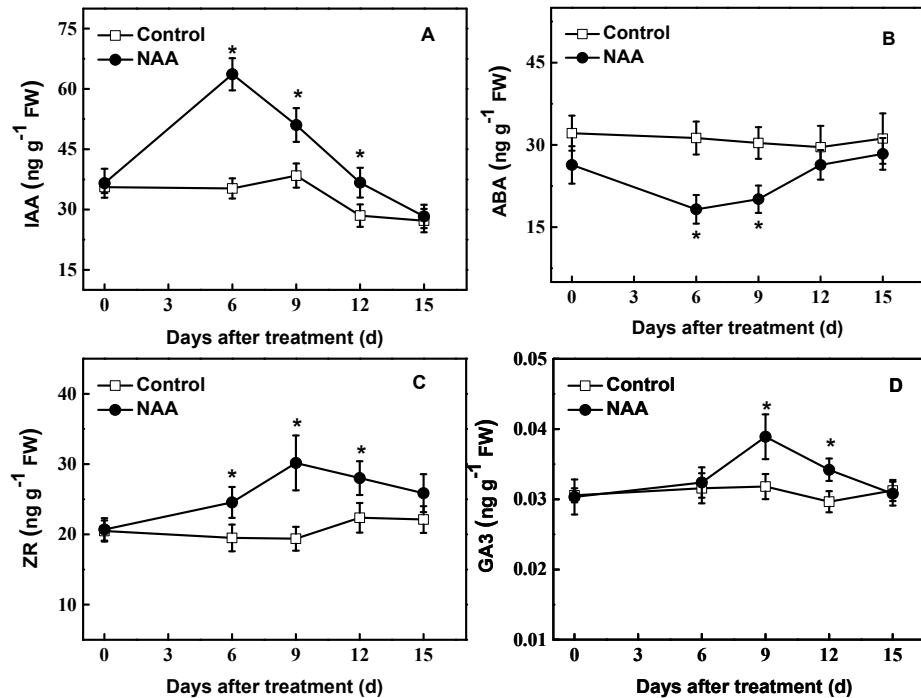


Figure 2 Endogenous levels of free IAA (A), ABA (B), ZR (C) and GA3 (D) in cuttings of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) treated with NAA (0.54 mM). Data are the means of at least five replicates with standard errors shown by vertical bars. * indicates significant differences at $p < 0.05$.

3.4. Biochemical changes during rooting

By contrast with endogenous IAA level, the IAAO activity in NAA-treated cuttings was decreased gradually during the induction phase of roots. At the end of 9th day, the IAAO activity in the NAA-treated tissues decreased by 25% compared with controls. The IAAO activity of NAA-treated cuttings increased gradually as the incubation was prolonged (Figure 3A). The POD activity of the NAA-treated cuttings was also lower than that of controls during the test period. On the 9th d, there was a significant decline ($P \leq 0.05$) in POD activity in NAA-treated tissues (67% of the control, Figure 3B). As the incubation time was prolonged, POD activity in NAA-treated cuttings increased, and the difference between NAA-treated and control tissues became less significant ($P \leq 0.05$). Unlike IAAO and POD, the PPO activity in NAA-treated tissues was highest at 6th d tested and then declined, but still higher than controls by the end of investigation (Figure 3C).

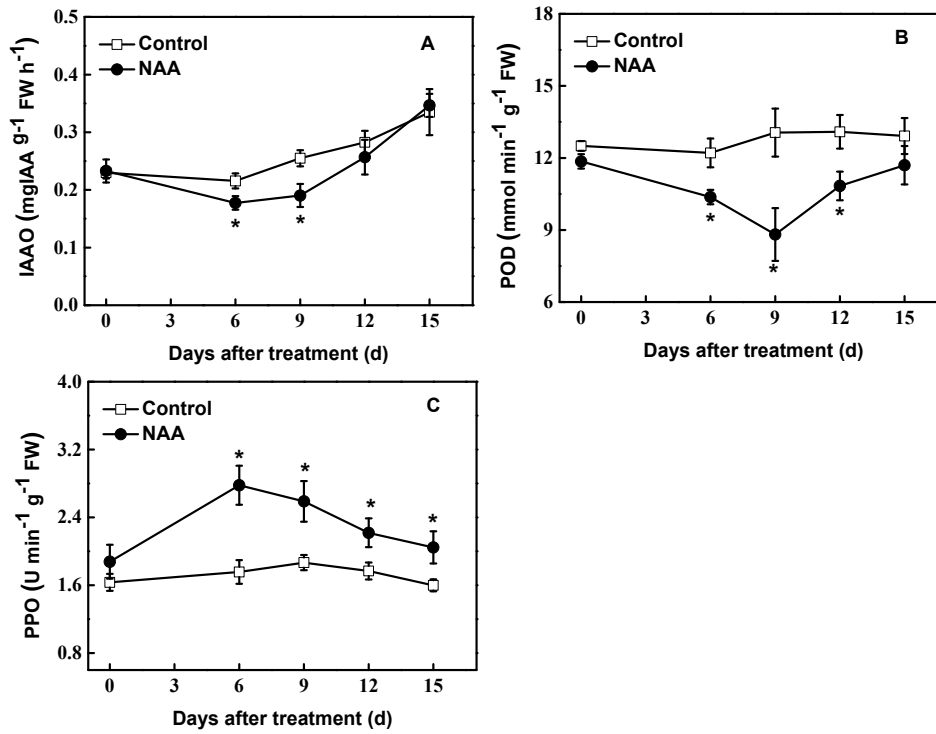


Figure 3 Effect of NAA on IAAO (A), PPO (B) and POD (C) activity in hybrid aspen (*Populus tremula* L.×*P. tremuloides* Michx.) cuttings. Data are the means of at least five replicates with standard errors shown by vertical bars. * indicates significant differences at p<0.05.

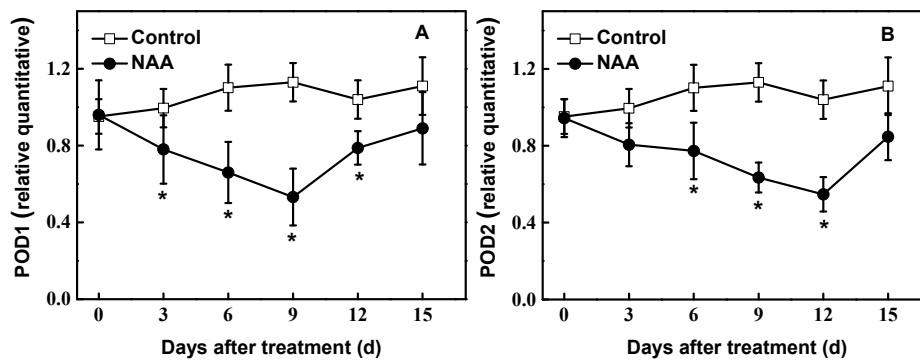


Figure 4 Effects of NAA on expression pattern of POD1 (a) and POD2 (b) in cuttings of hybrid aspen (*Populus tremula* L.×*P. tremuloides* Michx.) by qRT-PCR. Data are the means of at least five replicates with standard errors shown by vertical bars. * indicates significant differences at p<0.05.

3.5. Gene expression during rooting

Figure 4 shows the expression characteristics of POD1 and POD2 of cuttings exposed to 0.54 mM NAA and water (Control) for 15 days, respectively. We found that expression of POD1 was down-regulated gradually in NAA-treated tissues at the early examined period. Up to 9 d tested, the expression of POD decreased by 53% compared to control plants. For POD2, consistently with POD activity, it was significantly down-regulated, reaching 53% of controls at 12 d after NAA treatment (Figure 4).

4. Discussion

Adventitious root formation is often a limiting step for in vitro vegetative plant propagation programmes. As a lot of species, woody plants are difficult to form adventitious roots. The results of the present investigation indicated that it is effective to root by stem cuttings of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) with the application of auxins (Table 1). Auxins are well known to play a significant role in stimulating AR from stem cuttings of tree species [23-26]. In addition to enhancing the rate of AR development, auxin application has been found to increase the number of roots initiated per rooted cutting in a variety of species [27-28]. Among the growth hormones, NAA at 0.54 mM was superior in terms of rooting rate and number of rooting (per cutting) (Table 1) and hence it was recommended for rooting in hybrid aspen in our study. This is line with the fact that a particular type of auxin is effective in enhancing rooting in a particular species [29]. Similar trend was also recently recorded for hormonal applications at different concentrations by Tiwari and Das [30] who reported that NAA are known to promote the expansion of roots in cutting thereby increasing the survival rate. It is worth noting that higher concentrations of auxin did not substantially produce a better results of rooting. For example, cuttings treated with 2.7 mM NAA rooted worse than those with 0.54 or 1.62 mM NAA (Table 1). The inhibitory effect caused by high exogenous auxin also occurred in other plants such as peach [31] and whip grass [32].

In the present study, NAA was also proved to be efficient by morphological and anatomical observation of rooting process (Figure 1), with the same as in apple microcuttings [35], *Petunia hybrida* [36] and *Fuchsia hybrida* cuttings [37]. On the other hand, the timing of root initiation may be critical in determining the optimal application time for hormones [33,34]. The adventitious root primordia and root emergence were evident by 12 days in those treated cuttings with NAA (Figure 1) in our experiment, similar to the time of root emergence in cuttings of *Eucalyptus globulus* × *E. maidenii* [38] and of eucalypt hybrids [39].

Endogenous plant hormone levels in cuttings have been associated with root formation [40]. In the present study, NAA-treated cuttings showed an increase in endogenous IAA levels (Figure 2A). It is generally known that high concentrations of endogenous IAA has an important role in root initiation. In analogy to our results, higher IAA contents were found in basal shoot parts of *Ulmus glabra* Huds [41] and *Centaureum erythraea* [42]. In addition to IAA content, we also found that the activity of IAAO was reduced remarkably (Figure 3A), which might correspond to the rise in endogenous IAA levels, confirming previous results [43]. This suggests that the reduction of IAAO may be necessary for adventitious root formation [44]. In contrast to IAA, ABA content decreased after the cuttings were exposed to NAA treatment (Fig. 3B). Generally, low concentrations of ABA positively affect callus growth and organogenesis, but high concentrations of ABA have a negative effect [45, 46]. NAA treatment also induced the other endogenous hormones such as ZR and GA₃ (Figure 2C, D) to different extents during the investigated period. The levels of the ZR increased during the rooting period, which was thought to be related to the formation of the cortex root [47]. Yan et al. [48] suggested that the level of GA₃ was not changed or declined slightly in the initial cutting period, but increased markedly when calli were formed and root primordial differentiated. However, the changes in the levels of endogenous hormones were very complex, and many contrasting results were reported [49,50,42], which was most likely due to plant species, duration investigated, position of plants, environment factors, etc. Furthermore, it can be conjecturable that exogenously applied auxin possibly alter the concentrations of endogenous hormones, thus the original balance among endogenous hormones is replaced by the new balance beneficial to root formation [51].

Biochemical make-up plays a major role during rooting process in plant [52]. Except the changes of IAAO activity mentioned above, PPO activity was increased in NAA-treated cuttings during experimental phase (Figure 3C). The higher PPO activity might be associated with better rooting ability in treated cuttings, which was also consistent with data reported by Rout [32, 52]. POD are known to be involved in auxin metabolism as well as lignification processes in the cell wall in the presence of phenol [53,54], and our data showed that the decrease of POD activity in NAA-treated tissues (Figure 3B) may cause greater accumulation of endogenous IAA, and enhance the induction

of adventitious roots in NAA-treated hypocotyls [44]. Moncousin et al. [55] reported that low POD corresponded to high IAA levels before the visible appearance of roots. The decrease of POD activity is correlated with a reduction of the transcript levels of POD1 and POD2 (Figure 4), indicating that the decrease in the activity of POD is most likely due to the inhibition of the *de novo* synthesis of POD1 and POD2 during adventitious root induction. Klotz and Lagrimini [56] reported that NAA strongly suppressed POD gene expression by regulating the multiple auxin responsive elements within the POD gene promoter. Once POD gene expression is suppressed by NAA, which would result in the increased level of endogenous IAA, NAA worked together with endogenous IAA to stimulate cell division and elongation in treated tissues during the induction of adventitious roots in turn [57,58].

Consequently, the overall the rooting was best with 0.54 mM NAA in our study. The rooting response due to exogenous application of NAA was also reflected in the metabolic changes during adventitious root formation in cuttings. Treatment with NAA increased the levels of endogenous IAA, ZR and GA3 but reduced the level of ABA. Higher activities of IAAO and lower POD and PPO activity in the NAA treated cuttings were also found in cuttings with 0.54 mM NAA.

Acknowledgments: This work was supported by Fundamental Research Funds for the Central Universities (2572015DA02), the National Natural Science Foundation of China (30800876, 31170569) and the Innovation Project of State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University)

Author Contributions: Xing Shun Song and Shao Peng Yan conceived and designed the experiments; Shao Peng Yan performed the experiments; Rui Hua Yang performed the microscopy; Fang Wang and Li Na Sun analyzed the data; Xing Shun Song wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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